


JC07 Rec'd PCT/PTO 25 FEB 2002

EXPRESS MAIL MAILING LABEL NO. EL934376458US

FORM PTO-1390 (REV. 12-2001)		U.S. DEPARTMENT OF COMMERCE PATENT AND TRADEMARK OFFICE		ATTORNEY'S DOCKET NUMBER MIT-108	
TRANSMITTAL LETTER TO THE UNITED STATES DESIGNATED/ELECTED OFFICE (DO/EO/US) CONCERNING A FILING UNDER 35 U.S.C. 371				U.S. APPLICATION NO. (If known, see 37 CFR 1.5) 10/069280	
INTERNATIONAL APPLICATION NO. PCT/US00/23288		INTERNATIONAL FILING DATE 25 August 2000		PRIORITY DATE CLAIMED 25 August 1999	
TITLE OF INVENTION Pharmaceutical Preparations For The Inhibition of Herpes Simplex Virus 1 Entry					
APPLICANT(S) FOR DO/EO/US Shukla et al.					
Applicant herewith submits to the United States Designated/Elected Office (DO/EO/US) the following items and other information:					
<ol style="list-style-type: none"> 1. <input checked="" type="checkbox"/> This is a FIRST submission of items concerning a filing under 35 U.S.C. 371. 2. <input type="checkbox"/> This is a SECOND or SUBSEQUENT submission of items concerning a filing under 35 U.S.C. 371. 3. <input checked="" type="checkbox"/> This is an express request to begin national examination procedures (35 U.S.C. 371(f)). The submission must include items (5), (6), (9) and (21) indicated below. 4. <input checked="" type="checkbox"/> The US has been elected by the expiration of 19 months from the priority date (Article 31). 5. <input checked="" type="checkbox"/> A copy of the International Application as filed (35 U.S.C. 371(c)(2)) <ol style="list-style-type: none"> a. <input type="checkbox"/> is attached hereto (required only if not communicated by the International Bureau). b. <input checked="" type="checkbox"/> has been communicated by the International Bureau. (copy of Form PCT/IB/308 attached) c. <input type="checkbox"/> is not required, as the application was filed in the United States Receiving Office (RO/US). 6. <input type="checkbox"/> An English language translation of the International Application as filed (35 U.S.C. 371(c)(2)). <ol style="list-style-type: none"> a. <input type="checkbox"/> is attached hereto. b. <input type="checkbox"/> has been previously submitted under 35 U.S.C. 154(d)(4). 7. <input checked="" type="checkbox"/> Amendments to the claims of the International Application under PCT Article 19 (35 U.S.C. 371(c)(3)) <ol style="list-style-type: none"> a. <input type="checkbox"/> are attached hereto (required only if not communicated by the International Bureau). b. <input type="checkbox"/> have been communicated by the International Bureau. c. <input type="checkbox"/> have not been made; however, the time limit for making such amendments has NOT expired. d. <input checked="" type="checkbox"/> have not been made and will not be made. 8. <input type="checkbox"/> An English language translation of the amendments to the claims under PCT Article 19 (35 U.S.C. 371 (c)(3)). 9. <input type="checkbox"/> An oath or declaration of the inventor(s) (35 U.S.C. 371(c)(4)). 10. <input type="checkbox"/> An English language translation of the annexes of the International Preliminary Examination Report under PCT Article 36 (35 U.S.C. 371(c)(5)). 					
Items 11 to 20 below concern document(s) or information included:					
<ol style="list-style-type: none"> 11. <input type="checkbox"/> An Information Disclosure Statement under 37 CFR 1.97 and 1.98. 12. <input type="checkbox"/> An assignment document for recording. A separate cover sheet in compliance with 37 CFR 3.28 and 3.31 is included. 13. <input checked="" type="checkbox"/> A FIRST preliminary amendment. 14. <input type="checkbox"/> A SECOND or SUBSEQUENT preliminary amendment. 15. <input type="checkbox"/> A substitute specification. 16. <input type="checkbox"/> A change of power of attorney and/or address letter. 17. <input type="checkbox"/> A computer-readable form of the sequence listing in accordance with PCT Rule 13ter.2 and 35 U.S.C. 1.821 - 1.825. 18. <input type="checkbox"/> A second copy of the published international application under 35 U.S.C. 154(d)(4). 19. <input type="checkbox"/> A second copy of the English language translation of the international application under 35 U.S.C. 154(d)(4). 20. <input checked="" type="checkbox"/> Other items or information: Patent Data Entry Form with names and addresses of inventors Copy of Form PCT/IB/308 					

U.S. APPLICATION NO. (known) 10/069280 INTERNATIONAL APPLICATION NO. PCT/US00/23288		ATTORNEY'S DOCKET NUMBER MIT-108	
21. <input checked="" type="checkbox"/> The following fees are submitted: BASIC NATIONAL FEE (37 CFR 1.492 (a) (1) - (5)): Neither international preliminary examination fee (37 CFR 1.482) nor international search fee (37 CFR 1.445(a)(2)) paid to USPTO and International Search Report not prepared by the EPO or JPO..... \$1040.00 International preliminary examination fee (37 CFR 1.482) not paid to USPTO but International Search Report prepared by the EPO or JPO \$890.00 International preliminary examination fee (37 CFR 1.482) not paid to USPTO but international search fee (37 CFR 1.445(a)(2)) paid to USPTO \$740.00 International preliminary examination fee (37 CFR 1.482) paid to USPTO but all claims did not satisfy provisions of PCT Article 33(1)-(4) \$710.00 International preliminary examination fee (37 CFR 1.482) paid to USPTO and all claims satisfied provisions of PCT Article 33(1)-(4) \$100.00 ENTER APPROPRIATE BASIC FEE AMOUNT =		CALCULATIONS PTO USE ONLY	
		\$ 890.00	
Surcharge of \$130.00 for furnishing the oath or declaration later than <input type="checkbox"/> 20 <input type="checkbox"/> 30 months from the earliest claimed priority date (37 CFR 1.492(e)).		\$ -0-	
CLAIMS	NUMBER FILED	NUMBER EXTRA	RATE
Total claims	24 - 20 =	4	x \$18.00
Independent claims	1 - 3 =	0	x \$84.00
MULTIPLE DEPENDENT CLAIM(S) (if applicable)		+ \$280.00	\$ -0-
TOTAL OF ABOVE CALCULATIONS =			\$ 962.00
<input type="checkbox"/> Applicant claims small entity status. See 37 CFR 1.27. The fees indicated above are reduced by 1/2.		+	\$
SUBTOTAL =			\$ 962.00
Processing fee of \$130.00 for furnishing the English translation later than <input type="checkbox"/> 20 <input type="checkbox"/> 30 months from the earliest claimed priority date (37 CFR 1.492(f)).			\$
TOTAL NATIONAL FEE =			\$
Fee for recording the enclosed assignment (37 CFR 1.21(h)). The assignment must be accompanied by an appropriate cover sheet (37 CFR 3.28, 3.31). \$40.00 per property +			\$
TOTAL FEES ENCLOSED =			\$ 962.00
		Amount to be refunded:	\$
		charged:	\$
a. <input checked="" type="checkbox"/> A check in the amount of \$ <u>962.00</u> to cover the above fees is enclosed. b. <input type="checkbox"/> Please charge my Deposit Account No. _____ in the amount of \$ _____ to cover the above fees. A duplicate copy of this sheet is enclosed. c. <input checked="" type="checkbox"/> The Commissioner is hereby authorized to charge any additional fees which may be required, or credit any overpayment to Deposit Account No. <u>20-0531</u> . A duplicate copy of this sheet is enclosed. d. <input type="checkbox"/> Fees are to be charged to a credit card. WARNING: Information on this form may become public. Credit card information should not be included on this form. Provide credit card information and authorization on PTO-2038.			
NOTE: Where an appropriate time limit under 37 CFR 1.494 or 1.495 has not been met, a petition to revive (37 CFR 1.137 (a) or (b)) must be filed and granted to restore the application to pending status.			
SEND ALL CORRESPONDENCE TO: Patent Administrator Testa, Hurwitz & Thibault, LLP 125 High Street Boston, Massachusetts 02110-2704		 SIGNATURE Maureen A. Bresnahan NAME 44,559 REGISTRATION NUMBER	

EXPRESS MAIL MAILING LABEL NO. EL934376458US
PATENT
Attorney Docket No. MIT-108

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

APPLICANTS: Shukla et al.
SERIAL NO.: Not yet assigned GROUP NO.: Not yet assigned
FILING DATE: Herewith EXAMINER: Not yet assigned
I.A. SERIAL No.: PCT/US00/23288
I.A. FILING DATE: August 25, 2000
TITLE: PHARMACEUTICAL PREPARATIONS FOR THE
INHIBITION OF HERPES SIMPLEX VIRUS 1 ENTRY

BOX PCT
Commissioner for Patents
Washington, D.C. 20231

PRELIMINARY AMENDMENT

Sir:

Please make the following amendments to the patent application identified above before substantive examination.

AMENDMENT

In the Specification

On page 1, please insert the following paragraph after the title:

--Related Applications

This application is the national phase of International (PCT) Patent Application Serial No. PCT/US00/23288, filed August 25, 2000, which claims priority to U.S. Application No. 60/150,743, filed on August 25, 1999 and U.S. Application No. 60/175,347, filed on January 10, 2000, the disclosures of which are incorporated herein by reference.--

In the Claims

Please amend claims 3 and 12-24 to read as follows.

3. The pharmaceutical preparation of claim 1, comprising a polysaccharide preparation enriched for polysaccharide structures capable of specific binding with HSV-1 gD viral glycoprotein.
12. The pharmaceutical preparation of claim 1, wherein the preparation comprises the disaccharide sequence -IdoA2S-GlcN3S6S.
13. The pharmaceutical preparation of claim 1, wherein the preparation comprises the trisaccharide sequence GlcNS-IdoA2S-GlcNH₂3S6S.
14. The pharmaceutical preparation of claim 1, wherein the preparation comprises the tetrasaccharide sequence UA2S-GlcNS-IdoA2S-GlcNH₂3S6S.
15. The pharmaceutical preparation of claim 1, wherein the preparation comprises the pentasaccharide sequence GlcNS6S-UA2S-GlcNS-IdoA2S-GlcNH₂3S6S.
16. The pharmaceutical preparation of claim 1, wherein the preparation comprises the hexasaccharide sequence UA-GlcNS6S-UA2S-GlcNS-IdoA2S-GlcNH₂3S6S.
17. The pharmaceutical preparation of claim 1, wherein the preparation comprises the hexasaccharide sequence UA-GlcNS6S-UA2S-GlcNS-IdoA2S-GlcNH₂3S6S.
18. The pharmaceutical preparation of claim 1, wherein the preparation comprises the heptasaccharide sequence GlcNAc-UA-GlcNS6S-UA2S-GlcNS-IdoA2S-GlcNH₂3S6S.
19. The pharmaceutical preparation of claim 1, wherein the preparation comprises the octasaccharide sequence UA-GlcNAc-UA-GlcNS6S-UA2S-GlcNS-IdoA2S-GlcNH₂3S6S.
20. The pharmaceutical preparation of claim 1, wherein said pharmaceutical preparation comprises pharmaceutically acceptable carrier selected from the group consisting of lotions,

creams, jellies, liniments, ointments, salves, oils, foams, gels, washes, suppositories, slow-releasing polymers, and coatings.

21. The pharmaceutical preparation of claim 20, wherein said pharmaceutical preparation further comprises at least one skin penetrating enhancer.
22. The pharmaceutical preparation of claim 21, wherein said skin penetrating enhancer is selected from the group consisting of dimethylsulfoxide (DMSO), propylene glycol, isopropanol, ethanol, oleic acid, and N-methylpyrrolidone.
23. A method of inhibiting herpes simplex virus type-1 (HSV-1) viral infection in mammal comprising administering a therapeutically effective amount of the pharmaceutical preparation of claim 1, to a mammal at risk of HSV-1 infection.
24. A method of inhibiting herpes simplex virus type-1 (HSV-1) viral infection in mammal comprising administering a therapeutically effective amount of the pharmaceutical preparation of claim 1, to a mammal diagnosed with HSV-1 infection.

REMARKS


The instant application is the U.S. national phase of International Patent Application No. PCT/US00/23288, which was filed under the Patent Cooperation Treaty. Applicants hereby amend the application to better conform the application to U.S. patent practice.

Amendment to the Specification

Applicants have amended the specification to claim benefit of an earlier filing date and cross-reference related applications under 37 C.F.R. § 1.78. Applicants submit that no new matter is introduced by this amendment.

Amendment to the Claims

Applicants amended claims 3 and 12-24 to remove instances of multiple dependencies.

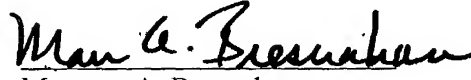


Entry of the foregoing amendments is respectfully requested. Applicants submit pending claims 1-24 are in condition for allowance and request an early favorable action.

Applicants believe no additional fees are necessitated by this Preliminary Amendment. However, in the event that any additional fees are due, the Commissioner is hereby authorized to charge any such fees relating to this submission to Deposit Account No. 20-0531.

Respectfully submitted,

Date: February 25, 2002
Reg. No. 44,559
Tel. No. (617) 248-7103
Fax No. (617) 248-7100


Maureen A. Bresnahan
Attorney for Applicants
Testa, Hurwitz & Thibault, LLP
High Street Tower
125 High Street
Boston, MA 02110

MARKED-UP VERSION OF AMENDED CLAIMS

3. The pharmaceutical preparation of claim 1 [claims 1 or 2], comprising a polysaccharide preparation enriched for polysaccharide structures capable of specific binding with HSV-1 gD viral glycoprotein.

12. The pharmaceutical preparation of claim 1 [claims 1-11], wherein the preparation comprises the disaccharide sequence -IdoA2S-GlcN3S6S.

13. The pharmaceutical preparation of claim 1 [claims 1-11], wherein the preparation comprises the trisaccharide sequence GlcNS-IdoA2S-GlcNH₂3S6S.

14. The pharmaceutical preparation of claim 1 [claims 1-11], wherein the preparation comprises the tetrasaccharide sequence UA2S-GlcNS-IdoA2S-GlcNH₂3S6S.

15. The pharmaceutical preparation of claim 1 [claims 1-11], wherein the preparation comprises the pentasaccharide sequence GlcNS6S-UA2S-GlcNS-IdoA2S-GlcNH₂3S6S.

16. The pharmaceutical preparation of claim 1 [claims 1-11], wherein the preparation comprises the hexasaccharide sequence UA-GlcNS6S-UA2S-GlcNS-IdoA2S-GlcNH₂3S6S.

17. The pharmaceutical preparation of claim 1 [claims 1-11], wherein the preparation comprises the hexasaccharide sequence UA-GlcNS6S-UA2S-GlcNS-IdoA2S-GlcNH₂3S6S.

18. The pharmaceutical preparation of claim 1 [claims 1-11], wherein the preparation comprises the heptasaccharide sequence GlcNAc-UA-GlcNS6S-UA2S-GlcNS-IdoA2S-GlcNH₂3S6S.

19. The pharmaceutical preparation of claim 1 [claims 1-11], wherein the preparation comprises the octasaccharide sequence UA-GlcNAc-UA-GlcNS6S-UA2S-GlcNS-IdoA2S-GlcNH₂3S6S.

20. The pharmaceutical preparation of claim 1 [claims 1-19], wherein said pharmaceutical preparation comprises pharmaceutically acceptable carrier selected from the group consisting of

lotions, creams, jellies, liniments, ointments, salves, oils, foams, gels, washes, suppositories, slow-releasing polymers, and coatings.

21. The pharmaceutical preparation of claim 1 [claims 1-20], wherein said pharmaceutical preparation further comprises at least one skin penetrating enhancer.

22. The pharmaceutical preparation of claim 20 [claims 1-21], wherein said skin penetrating enhancer is selected from the group consisting of dimethylsulfoxide (DMSO), propylene glycol, isopropanol, ethanol, oleic acid, and N-methylpyrrolidone.

23. A method of inhibiting herpes simplex virus type-1 (HSV-1) viral infection in mammal comprising administering a therapeutically effective amount of [any one of] the pharmaceutical preparation[s] of claim 1 [claims 1-22], to a mammal at risk of HSV-1 infection.

24. A method of inhibiting herpes simplex virus type-1 (HSV-1) viral infection in mammal comprising administering a therapeutically effective amount of [any one of] the pharmaceutical preparation[s] of claim 1 [claims 1-22], to a mammal diagnosed with HSV-1 infection.

PTO/PCT Rec'd 22 FEB 2002

**PHARMACEUTICAL PREPARATIONS FOR THE INHIBITION OF HERPES
SIMPLEX VIRUS 1 ENTRY****FIELD OF THE INVENTION**

The present invention relates generally to the field of virology. In particular, the present invention relates to methods and pharmaceutical preparations for inhibiting viral penetration and entry of herpes simplex virus 1 into mammalian cells via heparan sulfate binding.

BACKGROUND OF THE INVENTION

5 Heparan sulfate (HS) is widely expressed in animal and human tissues and has diverse roles in development, differentiation, and homeostasis. HS and other glycosaminoglycans are unbranched polymers covalently attached to the protein cores of proteoglycans, which are ubiquitously expressed as integral membrane proteins, glycerol phosphatidyl inositol-linked membrane proteins, and proteins of the extracellular matrix. The HS polymer is assembled by
10 sequential addition of D-glucuronic acid (GlcA) alternating with N-acetyl glucosamine (GlcNAc). The chains are then modified heterogeneously and in domains by de-acetylation and sulfation of glucosamine, epimerization of GlcA residues to iduronic acid (IdoA), and sulfation of hydroxyl groups. These modifications provide specific binding sites for a variety of proteins, including cell adhesion molecules, growth factors, chemokines, and factors regulating
15 angiogenesis and blood coagulation. Protein binding to HS may serve to sequester the protein at a particular site or to activate the protein. For example, the binding of antithrombin to a specific pentasaccharide sequence in HS results in activation of its anticoagulant activity (reviewed by Lindahl et al., 1998; Rosenberg et al., 1997).

 Herpes simplex viruses (HSV) are human herpesviruses of the neurotropic alpha
20 herpesvirus subfamily. Infections with HSV type 1 (HSV-1) and HSV type 2 (HSV-2) are highly prevalent. The usual manifestations of disease are mucocutaneous lesions of the mouth, face, eyes, or genitalia. Both HSV-1 and HSV-2 establish latent infections in neurons of peripheral ganglia and may reactivate to cause recurrent lesions. Rarely, the virus spreads to the central nervous system to cause meningitis or encephalitis (reviewed by Spear, 1993).

25 A number of viruses, including the human herpesviruses of the neurotropic alpha herpesvirus subfamily, use sites on HS as receptors for binding to cells (Byrnes and Griffin, 1998; Chung et al., 1998; Compton et al., 1993; Jackson et al., 1996; Shieh et al., 1992;

Summerford and Samulski, 1998). Viral entry may require interactions with other cell surface receptors as well (Geraghty et al., 1998; Montgomery et al., 1996; Summerford et al., 1999; Warner et al., 1998).

Infection involves (1) virus attachment to the cell surface membrane, followed by (2) virus penetration and entry into the cells. These two steps can be experimentally dissociated. In the case of HSV-1 and HSV-2, the virus binds to cells through interactions of cell surface HS with viral envelope glycoproteins gB and/or gC (reviewed by Spear, 1993). Certain cell types, such as swine testis or Chinese hamster ovary (CHO) cells, are resistant to viral entry, even though the viral binding to these cells appears normal. Following binding, a third viral envelope glycoprotein, gD, interacts with one of multiple specific receptors, resulting in viral entry by fusion of the virion envelope with the target cell membrane. This fusion reaction requires the concerted action of three additional viral glycoproteins, gB, gH and gL (reviewed by Spear, 1993), and appears to be triggered by the binding of gD to its cognate receptors.

The human gD-binding receptors that have been identified include a member of the TNF receptor family, designated HVEM (Montgomery et al., 1996; Whitbeck et al., 1997) or herpesvirus entry protein A (HveA), and officially named TNFRSF14, and two members of the immunoglobulin superfamily (Geraghty et al., 1998; Krummenacher et al., 1998; Warner et al., 1998). The latter two proteins are related to the poliovirus receptor (CD155) (Mendelsohn et al., 1989), were originally designated poliovirus receptor-related proteins 1 (Lopez et al., 1995) and 2 (Eberlé et al., 1995), and more recently have been referred to as HveC (Geraghty et al., 1998), and HveB (Warner et al., 1998) or nectin 1 and nectin 2 (Takahashi et al., 1999), respectively. Both HveA and HveC serve as gD-binding entry receptors for wild-type HSV-1 and HSV-2 strains, whereas HveB serves as an entry receptor for only a subset of HSV strains, (Geraghty et al., 1998; Montgomery et al., 1996; Warner et al., 1998). These gD-binding cell surface receptors are expressed at different levels in various human tissues and cell lines, suggesting a specific susceptibility to HSV in certain tissues.

It has been demonstrated that heparan sulfate 3-O-sulfotransferases (3-OSTs) are present as several isoforms (i.e., 3-OST-1, 3-OST-2, 3-OST-3, 3-OST-4) that are expressed at different levels in various tissues and cells (Shworak et al., 1999). In particular, 3-OST-3 enzymes are present in two highly similar forms (3-OST-3A and 3-OST-3B). In general, different 3-OST isoforms sulfate glucosamine residues with different saccharide sequences around the modification site. For example, 3-OST-1, 3-OST-2, and 3-OST-3 generate different saccharide sequences (Liu et al., 1999a). However, the highly homologous isoforms, 3-OST-3A and 3-

OST-3B, have been shown to sulfate identical saccharide sequences. The differences in sulfation of saccharide sequences are thought to correlate with differences in function. For example, heparan sulfates, which have been 3-O-sulfated by 3-OST-1, possess anticoagulant activity, however, heparan sulfates which have been 3-O-sulfated by 3-OST-2 and 3-OST-3A do not possess anticoagulant activity.

It has been shown that 3-OST-3s sulfate N-unsubstituted glucosamine residues (Liu et al., 1999b). However the majority of the glucosamine residues in HS are present either in the form of N-acetylated or N-sulfated glucosamine, and N-unsubstituted glucosamine residues are relatively rare. In fact, N-unsubstituted glucosamine residues constitute only 1-7 % of total glucosamine residues (Lindahl et al., 1998). The rarity of the saccharide sequences 3-O-sulfated by the 3-OST-3s, the multiplicity of the 3-OST-3 genes in human genome, and the correlation between sulfation and function, suggest an important biological role of 3-OST-3 modified HS.

SUMMARY OF THE INVENTION

The present invention depends, in part, on the discovery that specific saccharide sequences in HS can serve as a gD-binding receptor for HSV-1 entry into cells. Thus, the present invention provides pharmaceutical preparations for inhibiting HSV-1 infection, and methods of inhibiting HSV-1 infection in a mammal using the pharmaceutical preparations of the invention.

Thus, in one aspect, the present invention provides a pharmaceutical preparation for inhibiting HSV-1 infection in a mammal, comprising a polysaccharide preparation enriched for 3-O-sulfated glucosamine residues (GlcN3S and/or GlcN3S6S). In preferred embodiments, the polysaccharide preparation is enriched for polysaccharide sequences capable of specific binding with HSV-1 gD viral glycoprotein, for example, IdoA2S-GlcN3S6S and/or IdoA2S-GlcN3S.

In another series of embodiments, the polysaccharide preparation is enriched for a polysaccharide sequence that includes the disaccharide IdoA2S-GlcNH₂3S6S. In preferred embodiments, the polysaccharide preparation is enriched for the trisaccharide GlcNS-IdoA2S-GlcNH₂3S6S, the tetrasaccharide UA2S-GlcNS-IdoA2S-GlcNH₂3S6S the pentasaccharide GlcNS6S-UA2S-GlcNS-IdoA2S-GlcNH₂3S6S, the hexasaccharide UA-GlcNS6S-UA2S-GlcNS-IdoA2S-GlcNH₂3S6S, heptasaccharide GlcNAc-UA-GlcNS6S-UA2S-GlcNS-IdoA2S-GlcNH₂3S6S, and/or the octasaccharide UA-GlcNAc-UA-GlcNS6S-UA2S-GlcNS-IdoA2S-GlcNH₂3S6S. In a most preferred embodiment, the polysaccharide preparation is enriched for the octasaccharide UA-GlcNAc-UA-GlcNS6S-UA2S-GlcNS-IdoA2S-GlcNH₂3S6S.

In one series of embodiments, the polysaccharide sequence specifically binds to HSV-1 gD with a high degree of affinity. Thus, in one embodiment, the polysaccharide preparation has

a binding affinity (expressed in terms of the disassociation constant (K_d) and measured by affinity co-electrophoresis as described herein) of less than 30 μM for HSV-1 viral protein gD. In another embodiment, the polysaccharide structure has a binding specificity of less than 20 μM for HSV-1 viral protein gD. In a preferred embodiment, the polysaccharide structure has a binding specificity of less than 10 μM for HSV-1 viral protein gD. In a most preferred embodiment, the polysaccharide structure has a binding specificity of approximately 2 μM -10 μM for HSV-1 viral protein gD.

In another aspect, the present invention provides a pharmaceutical preparation for inhibiting HSV-1 infection in a mammal, including a polysaccharide that has been enriched for saccharide structures modified by a 3-OST-3 enzyme. In preferred embodiments the 3-OST-3 enzyme may be human 3-OST-3A or 3-OST-3B, or murine 3-OST-3B. Such enriched preparations may be produced by contacting a polysaccharide with a 3-OST-3 enzyme in the presence of a sulfate donor, under conditions suitable for 3-O sulfation of the polysaccharide by a 3-OST-3. In preferred embodiments, the polysaccharide preparation includes 3-O-sulfated heparan sulfate. In particularly preferred embodiments, the 3-O-sulfated heparan sulfate includes disaccharide sequences of IdoA2S-GlcN3S6S and/or IdoA2S-GlcN3S. In some embodiments the enriched preparation may be further enriched by purification of the 3-OST-3 modified polysaccharide by a purification technique that is known in the art, for example HPLC.

In another aspect, the pharmaceutical preparations of the present invention include a pharmaceutically acceptable carrier. In preferred embodiments, the carrier may be a lotion, cream, jelly, liniment, ointment, salve, oil, foam, gel, wash, suppository, slow-releasing polymer, or coating. In other embodiments, the preparation of the present invention may include at least one skin penetrating enhancer. In preferred embodiments, the skin penetrating enhancer may be dimethylsulfoxide (DMSO), propylene glycol, isopropanol, ethanol, oleic acid, and/or N-methylpyrrolidone.

In another aspect, the present invention provides for methods of inhibiting HSV-1 infection in a mammal, using the pharmaceutical preparations of the invention.

BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1 depicts a sequence alignment of the amino acid sequences of m3-OST-3B, h3-OST-3B, and the sulfotransferase domain of h3-OST-3A (amino acids 148-406).

Figure 2 depicts the IdoA2S-GlcNH $2\pm$ 6S3S substrate specificity of heparan sulfate D-glucosaminyl 3-O-sulfotransferase (3-OST-3).

DETAILED DESCRIPTION OF THE INVENTION

Definitions

In order to more clearly and distinctly point out and describe the subject matter that applicants regard as the invention, the following definitions are provided for certain terms used in the following written description and the appended claims.

Enriched As used herein with regard to particular polysaccharide structures within a polysaccharide preparation, the term "enriched" means that the proportion of the polysaccharide structure in a polysaccharide preparation is statistically significantly greater than the proportion of the polysaccharide structure in naturally-occurring, untreated polysaccharide preparation. The polysaccharide preparations of the invention are enriched for 3-OST-3-sulfated approximately 10-100 fold. Whereas the percentage of 3-OST-3-sulfated polysaccharide in a typical, unenriched preparation is less than 0.1%, the percentage of 3-OST-3-sulfated polysaccharide in the enriched polysaccharide preparations of the invention is about 5-7 %.

Heparan sulfate. As used herein, the term "heparan sulfate" or the abbreviation "HS" means a polysaccharide made up of repeated disaccharide units D-glucuronic acid or L-iduronic acid linked to N-acetyl or N-sulfated D-glucosamine. The polysaccharide is modified to a variable extent by sulfation of the 2-O-position of GlcA and IdoA residues, and the 6-O- and 3-O- positions of GlcN residues and acetylation or de-acetylation of the nitrogen of GlcN residues. Therefore, this definition encompasses all of the glycosaminoglycan compounds variously referred to as heparan(s), heparan sulfate(s), heparin(s), heparin sulfate(s), heparitin(s), heparitin sulfate(s), heparanoid(s), heparosan(s). The heparan molecules may be pure glycosaminoglycans or can be linked to other molecules, including other polymers such as proteins, and lipids, or small molecules such as biotin.

Heparan Sulfate D-Glucosaminyl 3-O-Sulfotransferases. Members of the family of 3-OSTs are responsible for the addition of sulfate groups at the 3-OH position of glucosamine in HS. These enzymes are present as several isoforms expressed from different genes at different levels in various tissues and cells, as described in WO 99/22005. The 3-OSTs act to modify HS late in its biosynthesis (reviewed by Lindahl et al., 1998) and each isoform recognizes as substrate glucosamine residues in regions of the HS chain that have specific, but different, prior modifications, including epimerization and sulfation at other nearby positions (Liu et al., 1999). Thus, different 3-OSTs generate different potential protein-binding sites in HS.

The 3-OST-3 proteins have 3-O-sulfotransferase activity on polysaccharide sequences including the sequences IdoA2S-GlcN and IdoA2S-GlcN6S, and convert these polysaccharide

sequences to IdoA2S-GlcN3S or IdoA2S-GlcN3S6S, respectively. As used herein, the term "3-OST-3" is meant to include the 3-OST-3A and 3-OST-3B proteins, allelic or conservative substitution variants, and combinations thereof, and/or recombinantly produced fusions or chimeras thereof, as described in WO 99/22005.

Figure 1 shows the sequence alignment of m3-OST-3B in comparison with the sequence of h3-OST-3B and h3-OST-3A. Single-underlined residues include the predicted membrane-spanning region of m3-OST-3B and the double-underlined residues indicate the potential sites for addition of N-linked glycans. The filled triangle marks the beginning of the conserved sulfotransferase domain. The position in this conserved domain of the amino acid substitution in h3-OST-3A (K162A) that results in loss of enzymatic activity is indicated by the shading. Within the sulfotransferase domain, m3-OST-3B is 91% identical to both human 3-OST-3s; the remaining N-terminal portion of m3-OST-3B is 81% identical to that of h3-OST-3B and unrelated to h3-OST-3A.

Figure 2 shows the substrate specificities of two 3-OST-3 isoforms. Each enzyme isoform transfers sulfate to the 3-OH position of glucosamine residues in different disaccharide and oligosaccharide sequences. The arrows indicate the sulfated position within glucosamine by the enzyme, and the 3-O-sulfation is highlighted by rectangles. The abbreviated names for the disaccharides are listed under each chemical structure, where R represents H or SO₃.

Specific Binding. As used herein, "specific binding" means that the binding affinity between a ligand and a receptor is sufficient to initiate a specific biological response. With regard to the binding of viral entry protein and target cell surface polysaccharides, the binding affinity is considered specific when the binding affinity of viral entry protein to cell surface polysaccharides is sufficient to induce fusion. With regard to the binding of viral protein gD and 3-OST-3 modified polysaccharides, binding is considered non-specific where the disassociation constant K_d has a value of greater than 30 μM as measured by affinity co-electrophoresis. Furthermore, with regard to the binding of viral protein gD and 3-OST-3 modified polysaccharides, binding is considered specific where the disassociation constant K_d has a value of less than 30 μM as measured by affinity co-electrophoresis.

Substantially pure. As used herein with respect to polysaccharide preparations, the term "substantially pure" means a preparation which contains at least 60% (by dry weight) the polysaccharide of interest, exclusive of the weight of other intentionally included compounds. Preferably the preparation is at least 75%, more preferably at least 90%, and most preferably at least 99%, by dry weight the polysaccharide of interest, exclusive of the weight of other

intentionally included compounds. Purity can be measured by any appropriate method, e.g., column chromatography, gel electrophoresis, amino acid compositional analysis or HPLC analysis. If a preparation intentionally includes two or more different polysaccharides of the invention, a "substantially pure" preparation means a preparation in which the total dry weight of the polysaccharide of the invention is at least 60% of the total dry weight, exclusive of the weight of other intentionally included compounds. Preferably, for such preparations containing two or more polysaccharides of the invention, the total weight of the polysaccharides of the invention should be at least 75%, more preferably at least 90%, and most preferably at least 99%, of the total dry weight of the preparation, exclusive of the weight of other intentionally included compounds. Thus, if the polysaccharides of the invention are mixed with one or more other compounds (e.g., diluents, detergents, excipients, salts, sugars, lipids) for purposes of administration, stability, storage, and the like, the weight of such other compounds is ignored in the calculation of the purity of the preparation. Furthermore, when the polysaccharide is a proteoglycan, the protein component of the proteoglycan is excluded for purposes of calculating purity.

Inhibiting. As used herein with respect to viral infection, the term "inhibiting" generally refers to the ability of a pharmaceutical preparation to prevent entry of a virus in a cell. Inhibition may be measured by monitoring the expression of viral genes in cells or the number of viruses produced. The viral entry assay described herein is an example of an in vitro method of measuring viral entry, and hence, viral inhibition. As used herein with regard to both primary and recurring HSV infection, the term "inhibiting" means that the amount of virus entering a cell is decreased by a statistically significant measurable amount in a cell, culture, or organism treated with the polysaccharide preparations of the invention relative to an untreated cell, culture, or organism.

I. Role of 3-O-Sulfated HS in HSV-1 Entry

The present invention depends, in part, on the discovery that specific saccharide sequences in HS can serve as gD-binding receptors for HSV-1 entry into cells. These saccharide sequences include one of the disaccharides, -IdoA2S-GlcN3S6S- or -IdoA2S-GlcN3S-. These conclusions are based on evidence that (1) 3-OST-3s capable of introducing the final modification characteristic of these disaccharides can convert HSV-1 resistant cells to cells susceptible to HSV-1 entry, (2) the ability of the 3-OST-3s to confer susceptibility depends on the presence of the appropriate HS substrate and on enzymatic activity, (3) gD receptors are generated by the action of 3-OST3s, (4) membrane-bound and soluble gD competed with virus

for the receptors generated by 3-OST-3B, and (5) HS-binding gD receptors can be removed from cells by the action of heparinases.

II. Pharmaceutical Preparations

In one aspect, the present invention provides for substantially pure pharmaceutical
5 preparations for use in the treatment of HSV-1 infection. Such pharmaceutical preparations include a substantially pure preparation of glycosaminoglycans or proteoglycan polysaccharides, which have been enriched for saccharide sequences capable of specifically binding to the viral envelope protein gD. In a preferred embodiment, the preparation includes glycosaminoglycans or proteoglycan polysaccharides enriched for GlcN3S and/or GlcN3S6S. In a more preferred
10 embodiment, the preparation includes glycosaminoglycans or proteoglycan polysaccharides enriched for IdoA2S-GlcN3S6S and/or IdoA2S-GlcN3S.

In another series of embodiments, the polysaccharide preparation is enriched for a polysaccharide sequence that includes the disaccharide IdoA2S-GlcNH₂3S6S. In preferred
15 embodiments, the polysaccharide preparation is enriched for the trisaccharide GlcNS-IdoA2S-GlcNH₂3S6S, the tetrasaccharide UA2S-GlcNS-IdoA2S-GlcNH₂3S6S the pentasaccharide GlcNS6S-UA2S-GlcNS-IdoA2S-GlcNH₂3S6S, the hexasaccharide UA-GlcNS6S-UA2S-GlcNS-IdoA2S-GlcNH₂3S6S, heptasaccharide GlcNAc-UA-GlcNS6S-UA2S-GlcNS-IdoA2S-GlcNH₂3S6S, and/or the octasaccharide UA-GlcNAc-UA-GlcNS6S-UA2S-GlcNS-IdoA2S-GlcNH₂3S6S. In a most preferred embodiment, the polysaccharide preparation is enriched for
20 the octasaccharide UA-GlcNAc-UA-GlcNS6S-UA2S-GlcNS-IdoA2S-GlcNH₂3S6S.

In preferred embodiments, the enriched polysaccharide sequence is capable of specifically binding to the viral envelope protein gD. Thus, the binding affinity between the polysaccharide sequence and the gD is sufficient to initiate a specific biological response. With regard to the binding of gD viral entry protein and the enriched polysaccharide sequences, the binding affinity
25 is considered specific when the binding affinity of gD to the enriched polysaccharides is sufficient to induce fusion.

In particular embodiments, the binding is considered non-specific where the disassociation constant K_d has a value of greater than 30 μ M, as measured by affinity co-electrophoresis. Furthermore, with regard to the binding of viral protein gD and 3-OST-3
30 modified polysaccharides, binding is considered specific where the disassociation constant K_d has a value of less than 30 μ M, as measured by affinity co-electrophoresis. In one series of embodiments, the polysaccharide sequence specifically binds to HSV-1 gD with a high degree of affinity. Thus, in one embodiment, the polysaccharide preparation has a binding affinity

(expressed in terms of the disassociation constant (K_d) and measured by affinity co-electrophoresis as described herein) of less than 30 μM for HSV-1 viral protein gD. In another embodiment, the polysaccharide structure has a binding specificity of less than 20 μM for HSV-1 viral protein gD. In a preferred embodiment, the polysaccharide structure has a binding specificity of less than 10 μM for HSV-1 viral protein gD. In a more preferred embodiment, the polysaccharide structure has a binding specificity of less than 5 μM for HSV-1 viral protein gD. In a most preferred embodiment, the polysaccharide structure has a binding specificity of approximately 2 μM to 10 μM for HSV-1 viral protein gD.

The enriched polysaccharide preparations of the invention may be produced by contacting a polysaccharide preparation with a 3-OST-3 enzyme, for example, 3-OST-3A and/or 3-OST-3B, in the presence of a sulfate donor under conditions that permit 3-OST-3 enzymatic activity. In particular, the 3-OST-3 proteins may be m3-OST-3B, h3-OST-3A, h3-OST-3B, allelic or conservative substitution variants, combinations thereof, and/or recombinantly produced fusions or chimeras thereof, as described in WO 99/22005. In preferred embodiments, the sulfate donor is 3'-phospho-adenosine 5'-phosphosulfate (PAPS). The enriched polysaccharide preparations of the present invention may be further enriched by one of the many purification techniques currently known in the art. For example, one of ordinary skill in the art could affinity purify the polysaccharide preparations of the invention by applying the preparation to a column or other substrate to which 3-OST-3 protein or gD has been immobilized, washing away the unbound fraction of HS, and thereafter eluting the binding fraction.

In one embodiment, the proportion of the 3-OST-3-modified residues in a polysaccharide preparation is statistically significantly greater than the proportion of the polysaccharide structure in naturally-occurring, untreated polysaccharide preparation. In a preferred embodiment, the proportion 3-OST-3-modified residues are greater than 7 %.

In some embodiments, the enriched polysaccharide preparations of the present invention may be combined with one or more other compounds (e.g., diluents, detergents, excipients, stabilizers, salts, sugars, lipids, or proteins) for purposes of administration, stability, and/or storage. Thus, in some embodiments, the pharmaceutical preparations of the present invention are incorporated into conventional carriers, such as, for example, lotions, creams, jellies, liniments, ointments, salves, oils, foams, gels, washes, suppositories, slow-releasing polymers, coatings, and the like so that they can be easily applied topically in the present methods. The carriers may also include other ingredients such as, for example, pH modifiers, stabilizers, buffers, surfactants, moisturizers, colorants, thickeners, flavorings, fragrances, perfumes, and the

like. The pharmaceutical preparations of the present invention may also be used with conventional birth-control or safe-sex devices. For example, the pharmaceutical preparations could be incorporated into or used in conjunction with condoms (i.e., via lubricants applied to the interior and/or exterior surfaces), diaphragms, cervical caps, or similar products. The pharmaceutical preparations of the present invention could also, for example, be introduced into a bodily orifice by hand, via suppositories, or by using conventional tampon or syringe techniques. The method of administering or delivering the pharmaceutical preparation to the potential HSV-transmission site is not critical if an effective amount of the pharmaceutical preparation is delivered to the site in a timely manner. Preferably the formulations and/or method of delivering the pharmaceutical preparations allows the pharmaceutical preparations to remain in the appropriate area during HSV-1 contact in order to maximize effectiveness.

The pharmaceutical preparations of the invention may also be combined with a skin penetrating enhancer, for example, dimethylsulfoxide (DMSO), propylene glycol, isopropanol, ethanol, oleic acid, and N-methylpyrrolidone. In preferred embodiments, the pharmaceutical preparations of the invention are combined with substance(s) that is/are appropriate for topical application to mucocutaneous tissues (i.e., tissues of the nasal, oral, vaginal, and anal orifices).

III. Methods of Treatment

In another aspect, the present invention is directed to methods of treating a mammal, (e.g., a human) diagnosed with or at risk of contracting HSV-1 infection. The present invention provides for therapeutic intervention of or prophylaxis for HSV-1 infection by reducing entry of HSV-1 into a target cell. As detailed above, 3-OST-3 modified HS binds to viral envelope protein gD and may promote the fusion of the viral coat with the target cell. Treatment or prophylaxis of HSV-1 infection may be achieved by administering a therapeutically effective amount of the pharmaceutical preparations of the invention including enriched gD-binding polysaccharide preparations to a mammal diagnosed with or at risk of HSV-1 infection. The pharmaceutical preparations of the present invention are preferably provided to a mammal directly (e.g., locally, as by injection, implantation or topical administration to a tissue locus). The preferred mode of administration of pharmaceutical preparations of the invention is topical.

In one embodiment, the pharmaceutical preparations of the invention may be used to prevent the primary (first-episode) HSV-1 infection. For example, the pharmaceutical preparation of the invention may be topically applied to tissue that is at risk of exposure to HSV-1. Thus, the present invention provides for methods of treating HSV-1 infection by administering a therapeutically effective amount of the pharmaceutical preparations of the

invention to an area that may be exposed to HSV-1. In preferred embodiments, the topical administration of the pharmaceutical preparations of the present invention occurs prior to the potential exposure to HSV-1. Without being bound to any particular theory of the invention, it is contemplated that the likelihood of HSV-1 infection may be reduced by using the pharmaceutical preparations of the invention to competitively inhibit the binding of the viral envelope protein gD to 3-OST-3 modified HS on a target cell surface.

In another embodiment, the pharmaceutical preparations of the invention may be used to treat persistent (recurring) HSV-1 infection. Thus, the present invention provides for methods of treating HSV-1 infection by administering a therapeutic amount of the pharmaceutical preparations of the invention to an affected area, such as a lesion, to ameliorate the clinical manifestations of HSV-1 infection. In preferred embodiments, the topical administration of the pharmaceutical preparations of the present invention occurs at the first sign of a prodromal syndrome, a detectable subclinical manifestation (e.g., prodromal syndrome or HSV-1 shedding on mucosal surfaces detectable by PCR) and/or clinical manifestation (e.g., lesion formation) of recurrent HSV-1 infection. In most preferred embodiments, the administration of the pharmaceutical preparations of the present invention occurs at the first sign of a detectable subclinical (e.g., HSV-shedding on mucosal surfaces detected by PCR) and/or clinical manifestation (e.g., lesion formation) of recurrent HSV-1 infection and continues until the subclinical and/or clinical manifestations have subsided. Without being bound to any particular theory of the invention, it is contemplated that the frequency, intensity and/or duration of HSV-1 recurring lesions may be reduced by using the pharmaceutical preparations of the invention to competitively inhibit the binding of gD to 3-OST-3 modified HS.

IV. Materials and Methods

Cells and Viruses

Wild-type CHO cells and GAG-defective mutant cell lines (pgsA-745, a cell line that does not produce glycosaminoglycans (Esko et al., 1985); pgsD-677, a cell line that produces only chondroitin sulfate (Lidholt et al., 1992); and pgsF-17, a cell line that fails to modify the hexuronic acid residues of HS by the addition of 2-O-sulfate groups (Bai and Esko, 1996)) were grown in Ham's F12 medium supplemented with 10% fetal bovine serum. Wild-type virus strains used were HSV-1(KOS), HSV-1(Patton), HSV-1(F) and HSV-2(333). Mutant strains included HSV-1(KOS)Rid1 and Rid2 (Dean et al., 1994). Recombinant viruses carrying the lacZ gene of E. coli and capable of expressing β -galactosidase as a reporter of entry included HSV-

1(KOS)gL86 (Montgomery et al., 1996), HSV-1(KOS)tk12 and HSV-1(KOS)Rid1-tk12 (Warner et al., 1998).

Antibodies and purified recombinant proteins

Antibodies used included rabbit antiserum R7 specific for HSV gD (Isola et al., 1989),
5 the anti-gD mAb DL6 (Isola et al., 1989) and an anti-myc mAb (cat. no. R950-25, Invitrogen, Carlsbad, CA). Purified recombinant proteins included m3-OST-1 secreted from L cells (Liu et al., 1996), a secreted N-truncated form of h3-OST-3A (Liu et al. 199b) and two secreted C-truncated forms of gD-1 designated gD-1(306t) and gD-1(Δ 290-299t) (Nicola et al., 1996). The
10 h3-OST-3A, gD-1(306t), and gD-1(Δ 290-299t) were produced in insect cells from baculovirus vectors and purified as described in the references cited. The hybrid protein gD-1:Fc was produced by CHO cells transfected with plasmid pBG64. The hybrid protein was harvested from the medium (Ham's F12 supplemented with 3% fetal bovine serum depleted of IgG) and the protein was purified by Protein G-Sepharose chromatography.

Screening assay for cDNAs encoding HSV-1 entry proteins

15 Wild-type Chinese hamster ovary (CHO) cells are resistant to HSV-1 entry (Shieh et al., 1992). A 15.5 days gestation mouse embryonic cDNA expression library (Gibco-BRL, Gaithersburg, MD) was screened for plasmids capable of rendering transfected CHO cells susceptible to HSV-1 entry, as previously described (Montgomery et al., 1996). A single plasmid, designated pDS43, was obtained. This plasmid had an insert of 1666 base pairs,
20 including the polyA tail with one major open reading frame encoding the protein. The sequence of the insert was deposited in GenBank (Accession No. AF168992).

Searching the protein databases using the BLAST Algorithm with the pDS43 amino acid sequence revealed that the clone has 74% identity to 3-OST-3A (Accession No. AF105376) from Residue 31 to 390 and has 86% identity to h3-OST-3B (Accession No. AF105377) from Residue
25 24 to 390. Such high identity to human 3-OST-3A and 3-OST-3B led to the conclusion that pDS43 encodes a murine form of 3-OST-3. The clone was shown to have 3-OST-3 activity and was therefore designated m3-OST-3B.

Transfection of the plasmids expressing 3-OST-3A and 3-OST-3B resulted in CHO-K1 becoming susceptible to KOS infection, indicating that human 3-OST-3A and 3-OST3B contain
30 viral entry activity. However, CHO-K1 cells transfected with the plasmid expressing 3-OST-1, an isoform of 3-OST-3, remain resistant to viral infection. Transfection of the plasmid expressing 3-OST-2, which has 67% identity to m3-OST-3B from Residue 30 to 390, caused transfected CHO-K1 cells to be susceptible to viral infection.

Plasmids

Plasmid pDS43/myc-His was generated by in-frame fusion of the C-terminus of the m3-OST-3B open reading frame, through conversion of the stop codon to a BamHI site, with the myc-His tag from the plasmid pcDNA3.1/myc-His (Invitrogen, Carlsbad, CA). Plasmid pBG64 has the ectodomain of HSV-1(KOS) gD (amino acids 1-343) fused in frame to the Fc region of a rabbit IgG heavy chain, and cloned into pcDNA3. Plasmids described elsewhere include those expressing m3-OST-1 (Shworak et al., 1997), h3-OST-3A and h3-OST-3B (Liu et al., 1999), pBG38 expressing HveC (Geraghty et al., 1998), and pRE4 expressing HSV-1 gD (Cohen et al., 1988).

Degradation analysis of 3-OST-3-modified HS

Purified enzymes (m3-OST-1 or h3-OST-3A) or extracts of COS-7 cells (Shworak et al., 1997) expressing various forms of 3-OST-3 were incubated with HS prepared from wild-type CHO cells or pgsF-17 cells and [³⁵S]PAPS. The reaction conditions ensured the modification of all potential 3-O-sulfation sites recognized by each enzyme. In some cases the enzyme-modified or unmodified HS was then incubated with nitrous acid at pH 1.5 and 0°C for 30 min and reduced with sodium borohydride (0.05M) under alkaline conditions at 50°C for 30 min (Liu et al., 1999). The [³⁵S]disaccharides were isolated by Biogel P-2 (Indianapolis, IN) and analyzed on reverse phase ion pairing HPLC. The identities of the [³⁵S]disaccharides were determined by co-elution with [³H]disaccharide standards. The disaccharides (about 60% of total loaded ³⁵S counts) were subfractionated on reversed-phase ion pairing HPLC with appropriate disaccharide standards.

Viral Entry Assays

Entry assays were based on quantitation of β-galactosidase expressed from the viral genome or by CHO-IE8 cells in which β-galactosidase expression is inducible by HSV infection (Montgomery et al., 1996). Cells were transiently transfected in 6-well dishes, using Lipofectamine (Gibco-BRL, Gaithersburg, MD) with plasmids expressing one of the entry mediators or control plasmid, pcDNA3, at 1.5 to 2.0 μg per well in 1 ml. At 24 hours after transfection, the cells were replated in 96 well tissue culture dishes (2-4 X 10⁴ cells/well) at least 16 hours prior to infection. Cells were washed and exposed to virus in 50 μl of phosphate-buffered saline (PBS) containing glucose and 1% calf serum (PBS-G-CS) for 6 hours at 37°C before solubilization in 100μl of PBS containing 0.5% NP-40 and the β-galactosidase substrate, o-nitro-phenyl β-D-galactopyranoside (ONPG, 3 mg/ml). The enzymatic activity was monitored

by spectrometry at several time points after the addition of ONPG to define the interval over which the generation of product was linear with time (Dynatech ELISA reader or a Spectromax 250). Results are presented as the means of triplicate or quadruplicate determinations. Assays for inhibition of infection by soluble gD were similar, except that dilutions of virus were mixed with gD-1(Δ 290-299t) or BSA and incubated for 30 minutes at 37°C prior to addition of the mixtures (50 μ l/well) to washed cell monolayers.

gD Interference Assay

Wild-type CHO cells were co-transfected, using Lipofectamine (Gibco-BRL, Gaithersburg, MD), with m3-OST-3B expression plasmid (pDS43/myc-His) and gD expression plasmid (pRE4 for HSV-1 wild-type gD) or control plasmid (pcDNA3), in 1:4 (m3-OST-3B:gD or control) ratio, in 6-well dishes. The total amount of plasmid DNA added per well was 2.0 μ g. After 24 hrs the transfected cells were replated in 96 well plates and, 24 hrs later, were exposed to various doses of β -galactosidase-expressing HSV-1. Six hours later, viral entry was quantitated as described herein.

Binding of gD-1 to 3-O-sulfated HS

Metabolically-labeled [3 H]HS (2-4 x 10⁶ dpm, 1.2-2.5 pmole) from wild-type CHO cells or pgsF-17 cells was incubated with [35 S]PAPS (10 μ M, 20 x 10⁶ dpm) and 60 ng of purified m3-OST-1 or h3-OST-3A. The average number of 3-O-sulfate groups introduced per HS molecule from wild-type CHO cells was 8 for 3-OST-1 and 14 for h3-OST-3A, based on the 35 S count transferred to the HS, given the specific activities of [35 S]PAPS (18 Ci/mmol) and [3 H]HS (727 Ci/mmol), and assuming a molecular weight of 81,000 for HS. Each preparation of enzyme-modified or unmodified HS (1-2 x 10⁵ dpm of 3 H) was mixed with gD-1(306t) (10 μ g) in 50 μ l of binding buffer (50 mM Tris-HCl, pH 7.5, 150 mM NaCl and 0.01% Triton X-100) for 30 min at room temperature. The anti-gD antiserum R7 (or the anti-gD monoclonal antibody DL6) (5 μ l) was added for incubation at 4°C for 1 hr, followed by addition of Protein A-agarose gel (80 μ l of 1:1 slurry) and agitation at 4°C for an additional hour. The Protein A-agarose gel was washed with binding buffer and the bound HS eluted with 1 ml of binding buffer containing 1 M NaCl.

3-OST-3 modified HS binds gD with a Kd of 2 μ M

Affinity co-electrophoresis (Lee et al., 1991) was performed to estimate dissociation constants for the gD-HS interactions. [35 S]HS was purified from CHO cells, modified by incubation with purified h3-OST-3A in vitro and then fractionated by co-immunoprecipitation

with gD-1(306t). The fraction of HS co-precipitating with gD was designated $\text{HS}^{\text{gD}\bullet\text{Act}}$ and the non-gD-binding fraction was designated $\text{HS}^{\text{gD}\bullet\text{Inact}}$.

Metabolically-labeled [^{35}S]HS was modified by incubation with purified h3-OST-3A as above except that unlabeled PAPS (10 μM) was the sulfate donor. The molecules of modified [^{35}S]HS capable of binding gD-1 ($[\text{gD}\bullet\text{Act}]$) were collected by co-precipitation with gD-1(306t) as described above. The molecules that failed to co-precipitate were designated [^{35}S]HS $^{\text{gD}\bullet\text{Inact}}$ although this fraction probably contained some gD-1-binding material. Purified gD-1(306t) was cast in low melting point 1% agarose gel (Gibco-BRL, Gaithersburg, MD) in a buffer containing 125 mM sodium acetate, 50 mM 3-(N-morpholino)-2-hydroxypropanesulfonic acid and 0.5% 3-[(3-cholamidopropyl)dimethylammonio]-1-propane-sulfonate, pH 7.0, at 9 different concentrations for [^{35}S]HS $^{\text{gD}\bullet\text{Act}}$, ranging from 0 to 11 μM , and at 4 different concentrations for [^{35}S]HS $^{\text{gD}\bullet\text{Inact}}$, ranging from 0 to 83 μM , in each separation zone. Approximately 50,000 cpm of [^{35}S]HS $^{\text{gD}\bullet\text{Act}}$ or [^{35}S]HS $^{\text{gD}\bullet\text{Inact}}$ was loaded in each separation zone and electrophoresis was performed at 400 mA for 2.5 hr in a cold room with circulated cold water. The gels were dried and analyzed on a Beta-scope 603 (Betagen) to determine the migration of [^{35}S]HS.

The [^{35}S]- intensity was plotted against the migration distance through the separation zone, and the migration point with the highest [^{35}S] intensity was defined as the migration of [^{35}S]HS $^{\text{gD}\bullet\text{Act}}$. The retardation coefficient $R = (M_0 - M)/M_0$, where M_0 is the migration of free [^{35}S]HS $^{\text{gD}\bullet\text{Act}}$ and M is the observed migration of [^{35}S]HS $^{\text{gD}\bullet\text{Act}}$. Provided that [^{35}S]HS $^{\text{gD}\bullet\text{Act}}$ and gD-1 forms 1:1 complex and the amount of [^{35}S]HS $^{\text{gD}\bullet\text{Act}}$ is far less than gD-1, a plot of $R/[\text{gD-1}]_{\text{total}}$ vs. R should yield a straight line with a slope of $-1/K_d$ according to Scatchard equation. From these data, the K_d for interaction of HS $^{\text{gD}\bullet\text{Act}}$ with gD-1(306t) was calculated to be 2 μM and the K_d for HS $^{\text{gD}\bullet\text{Inact}}$ was calculated to be 43 μM or greater.

V. Experimental Results

3-OST-3-modified HS specifically binds glycoprotein gD-1

It was shown that gD binds to 3-OST-3-modified HS, but not to 3-OST-3 enzyme. HS extracted from wild-type CHO cells was unmodified or was sulfated by incubation in vitro with 3-OST-1 or h3-OST-3A, and was tested for binding to gD in co-immunoprecipitation experiments. Table 1 shows that the fraction of labeled HS bound to immunoprecipitated gD-1(306t) was enhanced almost 10-fold for HS by incubation with 3-OST-3A. Modification by 3-OST-1 did not significantly enhance the binding to gD-1(306t). After incubation with 3-OST-

3A, the HS from pgsF-17 cells, a cell line that fails to modify the hexuronic acid residues of HS by the addition of 2-O-sulfate groups, bound to gD-1(306t) with about the same efficiency as unmodified HS from CHO cells.

Table 1. The binding of enzyme-modified HS to gD-1

[³ H]HS	[³ H]HS bound to gD-1(306t) (%)	
	Wild-type	pgsF-17
Unmodified HS	1.7±0.9 (n=5)	0.13±0.1 (n=2)
3-OST-1 modified HS ^a	3.4±1.6 (n=2)	N.D.
3-OST-3A modified HS ^b	14.8±1.7 (n=6)	1.6±0.7 (n=2)

^a The sulfated structure generated is -GlcA-GlcNS±6S3S-.

^b The sulfated structure generated is -IdoA2S-GlcN±6S3S-.

Efforts to demonstrate direct interactions between 3-OST-3 and gD were unsuccessful. In ELISA assays no binding of purified gD-1(306t) to purified h3-OST-3A was evident, whereas binding to purified HveC was readily detected in control experiments.

3-OST-3B-mediated viral entry depends on HS production and 2-O-sulfation of HS

A variety of mutant cell lines defective in various aspects of GAG biosynthesis have been isolated from CHO cells (Bai and Esko, 1996; Esko, 1991). Wild-type CHO cells and several mutant cell lines were transfected with plasmids expressing myc-tagged version of m3-OST-3B or HveC, or with the empty vector, pcDNA3. The cells were then exposed to HSV-1(KOS)gL86 for quantitation of viral entry. Various cell lines transfected with the m3-OST-3B-expressing plasmid produced equivalent amounts of the protein. Mutations affecting HS biosynthesis had different consequences for HSV-1 entry, depending on whether the entry mediator was m3-OST-3B or HveC. None of the cells transfected with control plasmid was susceptible to HSV-1 entry.

Expression of m3-OST-3B resulted in HSV-1 entry only in cells capable of producing HS with the modifications characteristic of CHO cells. HSV-1 entry occurred in wild-type CHO cells and mutant cells pgsE-606, which produce HS that is under-sulfated due to the absence of one of the N-sulfotransferase isoforms (Aikawa and Esko, 1999; Bame and Esko, 1989), provided 3-OST-3 was expressed. However, HSV-1 entry did not occur in mutant lines that fail to produce HS. For example, HSV-1 entry did not occur in the following HS defective cell lines: pgsA-745, a cell line that does not produce glycosaminoglycans (Esko et al., 1985); pgsD-677, a cell line that produces only chondroitin sulfate (Lidholt et al., 1992); and pgsF-17, a cell line that fails to modify the hexuronic acid residues of HS by the addition of 2-O-sulfate groups (Bai and Esko, 1996). Because the substrate for 3-OST-3s is a glucosamine residue adjacent to 2-O-

sulfated IdoA, incubation of HS from pgsF-17 cells with purified 3-OST-3A should not yield the characteristic disaccharides resulting from 3-OST-3 activity, as was confirmed experimentally.

In contrast to the results obtained for transfection with m3-OST-3B, expression of HveC rendered all the cell lines susceptible to HSV-1 entry, although the wild-type CHO cells became significantly more susceptible than any of the mutant cell lines. Binding of virus to the wild-type cells should be enhanced by interactions of gB or gC with HS as well as of gD with HveC. The ability of HS to enhance the susceptibility resulting from HveC expression depended on the level of glucosamine N-sulfation (pgsE-606 cells) and on 2-O-sulfation of hexuronic residues (pgsF-17 cells), suggesting that these groups are important for the binding of virus to cells (Feyzi et al., 1997; Herold et al., 1996).

HveC can confer greater susceptibility to HSV-1 entry than does m3-OST-3B. In the case of HveC, new gD-binding receptors are over-expressed from the transfected plasmid whereas, in the case of m3-OST-3B, the enzyme is thought to modify endogenous molecules to generate receptors for virus. Accordingly, the levels of endogenous receptor are believed to control the degree of susceptibility to infection resulting from 3-OST-3 activity.

Heparinases removed gD-binding receptors and reduced viral entry in 3-OST-3 cells

Heparinases were shown to remove the receptors that bind gD and mediate viral entry from 3-OST-3-expressing cells. Wild-type CHO cells or pgsA-745 cells were transfected with plasmids expressing m3-OST-3B or h3-OST-3A, or with control plasmid. The transfected cells were either incubated with a mixture of heparinases I and III or in buffer without the heparinases. The cells were next incubated with a gD hybrid protein consisting of the ectodomain of gD fused to the Fc of rabbit IgG (gD-1:Fc).

Heparinase I (heparin lyase I; heparinase; EC 4.2.2.8) and heparinase III (heparitin sulfate lyase III; heparitinase, EC 4.2.2.8) were obtained from Sigma (St. Louis, MO). Transfected cells plated in 96-well dishes were washed twice with PBS lacking magnesium and calcium ions, overlaid with several dilutions of heparinase I and heparinase III or PBS alone, and incubated at 30°C for 2 h. The cells were then washed with PBS and used for viral entry assays or for quantitation of gD-1:Fc binding to the cells.

Following heparinase treatment, the cells assayed for gD-binding were processed as follows. The cells were next incubated with a gD hybrid protein consisting of the ectodomain of gD fused to the Fc of rabbit IgG (gD-1:Fc). Expression of 3-OST-3A or m3-OST-3B caused enhanced binding of gD-1:Fc. In contrast, expression of 3-OST-3A or m3-OST-3B in pgs-745

cells did not enhance binding of gD-1:Fc. The enhanced binding in CHO cells was eliminated by pre-treatment of the cells with heparinases I/III.

Following heparinase treatment, the cells assayed for viral entry were processed as follows. The cells were exposed to HSV-1(KOS)gL86 at 7.5×10^5 PFU/well and viral entry was quantitated 6 hrs later. Entry into the heparinase I/III-treated cells was compared to percentage of the entry into mock-treated cells. For CHO cells expressing m3-OST-3B, treatment with heparinases I/III almost completely eliminated susceptibility to HSV-1 entry whereas, for cells expressing HveC, this treatment reduced susceptibility to HSV-1 infection by 50%.

Both membrane bound and soluble gD competes with virus for receptors generated by m3-OST-3B

HSV-1 entry was inhibited in CHO cells transformed with HSV-1 gD and m3-OST-3B. CHO cells were transfected with a m3-OST-3B-expressing plasmid mixed in 1:4 ratio with a gD-1-expressing plasmid or the empty vector pcDNA3. The transfected cells were then exposed to HSV-1(KOS)gL86 and viral entry quantitated after 6 hrs. Viral entry was assayed using the entry assay described herein.

HSV-1 entry was inhibited in m3-OST-3B-expressing CHO cells that were pre-incubated with soluble gD. CHO cells transfected with a plasmid expressing m3-OST-3B were exposed to HSV-1(KOS)gL86 (7.5×10^5 PFU/well) that had been pre-incubated with gD-1($\Delta 290-299$ t), a soluble form of gD-1. Viral entry was assayed using the entry assay described herein.

h3-OST-3A K162 mutant does not express 3-OST-3 activity

A mutated form of h3-OST-3A, in which the Lys at position 162 was changed to Ala, was shown by Western blot analysis to be expressed at a level comparable to that of wild-type enzyme but to have little or no enzymatic activity and no viral entry activity, as described below. The 3-OST-3A mutant was transfected into COS-7 cells and the cell extract was assayed for 3-OST-3 activity. Two characteristic 3-OST-3 degradation product [35 S]disaccharides, IdoA2S-AnMan3S and IdoA2S-AnMan3S6S (corresponding to IdoA2S-GlcN3S and IdoA2S-GlcN3S6S, respectively) were absent from the [35 S]HS prepared by the cell extract transfected with 3-OST-3A mutant.

This particular amino acid substitution in a highly conserved region of the sulfotransferases was shown previously to dramatically reduce enzymatic activity of a flavonol 3-sulfotransferase with minimal effect on PAPS binding (Marsolais and Varin, 1995). Moreover, mutagenic and structural studies demonstrated that the conserved Lys has a comparable critical role in catalysis by a heparan N-sulfotransferase (Kakuta et al., 1999; Sueyoshi et al., 1998).

Because the Lys 162 residue is conserved in almost all known sulfotransferase, it is likely to be a critical residue for sulfotransferase activity.

Binding of enzyme-modified HS to viral glycoproteins

The binding of enzyme-modified HS to viral glycoproteins was determined by immunoprecipitation using Protein A-agarose (Pierce, Rockford, IL). Briefly, enzyme-modified [$^3\text{H}/^{35}\text{S}$]HS (1×10^5 cpm of ^3H counts) was incubated with recombinant purified HSV glycoprotein gD-1 (306t, 10 μg) (Tal-Singer et al., 1995) in 50 μl of a buffer containing 50 mM Tris, 150 mM NaCl and 0.01% Triton X-100, pH 7.5 (binding buffer) at room temperature for 30 min. Polyclonal antibody R7 (5 μl), against gD-1 (Isola et al., 1989), was added to the mixture, and the reaction was incubated at 4°C for one additional hour. The reaction mixture was then mixed with 80 μl 1:1 slurry of pre-washed Protein A-agarose, and agitated at 4°C for one hour or overnight. The suspended solution was centrifuged at 5,000 x g for 2 min, and the supernatant was discarded. The Protein A-agarose gel was washed with 1 ml of binding buffer three times. The bound [$^3\text{H}/^{35}\text{S}$]HS was eluted with 1 ml of binding buffer containing 1 M NaCl.

Detection of gD-1 receptors on cells

CHO cells and pgsA-745 cells were transfected with pDS43 or pcDNA3 and plated in 96-well plates. Some cultures were treated with heparinases as described below prior to the assay. After washing the cells and blocking with PBS containing 3% BSA for 30 min, the cells were incubated with purified gD-1:Fc (1 $\mu\text{g}/\text{ml}$) in 1% BSA-PBS for 45 min at room temperature. The cells were then washed and fixed with 2% formaldehyde and 0.2% glutaraldehyde in PBS. Cell-bound gD-1:Fc was detected by sequential incubations with biotin-conjugated goat anti-rabbit IgG antibody (Sigma, St Louis, MO), Amdex™ streptavidin-horseradish peroxidase (Amersham Pharmacia Biotech, Inc., Uppsala, Sweden) and the peroxidase substrate 3,3',5,5'-tetramethyl benzidine (Sigma, St. Louis, MO) in 50 mM phosphate-citrate buffer. Product was quantitated by use of a Spectromax 250 ELISA (Molecular Devices Sunnyvale, CA) reader to measure OD₃₇₀.

References

- Aikawa, J., and Esko, J. D. (1999). Molecular cloning and expression of a third member of the heparan sulfate/heparin N-deacetylase/N-sulfotransferase family. *J.Biol.Chem.* 274, 2690-2695.
- Bai, X., and Esko, J. D. (1996). An animal cell mutant defective in heparan sulfate hexuronic acid 2-O-sulfation. *J.Biol.Chem.* 271, 17711-17717.
- 5 Bame, K. J., and Esko, J. D. (1989). Undersulfated heparan sulfate in a Chinese hamster ovary cell mutant defective in heparan sulfate N-sulfotransferase. *J.Biol.Chem.* 264, 8059-8065.
- Byrnes, A. P., and Griffin, D. E. (1998). Binding of Sindbis virus to cell surface heparan sulfate. *J.Virol.* 72, 7349-7356.
- 10 Chung, C. S., Hsiao, J. C., Chang, Y. S., and Chang, W. (1998). A27L protein mediates vaccinia virus interaction with cell surface heparan sulfate. *J.Virol.* 72, 1577-1585.
- Cohen, G. H., Wilcox, W. C., Sodora, D. L., Long, D., Levin, J. Z., and Eisenberg, R. J. (1988). Expression of herpes simplex virus type 1 glycoprotein D deletion mutants in mammalian cells. *J.Virol.* 62, 1932-1940.
- 15 Compton, T., Nowlin, D. M., and Cooper, N. R. (1993). Initiation of human cytomegalovirus infection requires initial interaction with cell surface heparan sulfate. *Virology* 193, 834-841.
- Corey, L., and Spear, P. G. (1988). Infections with herpes simplex viruses. *N.Eng.J.Med.* 314, 686-689; 749-757.
- 20 Dean, H. J., Terhune, S., Shieh, M.-T., Susmarski, N., and Spear, P. G. (1994). Single amino acid substitutions in gD of herpes simplex virus 1 confer resistance to gD-mediated interference and cause cell type-dependent alterations in infectivity. *Virology* 199, 67-80.
- Eberlé, F., Dubreuil, P., Mattei, M. G., Devillard, E., and Lopez, M. (1995). The human PRR2 gene, related to the human poliovirus receptor gene (PVR), is the true homolog of the murine MPH gene. *Gene* 159, 267-272.
- 25 Esko, J. D. (1991). Genetic analysis of proteoglycan structure, function and metabolism. *Curr.Opin.Cell Biol.* 3, 805-816.
- Esko, J. D., Stewart, T. E., and Taylor, W. H. (1985). Animal cell mutants defective in glycosaminoglycan biosynthesis. *Proc.Natl.Acad.Sci.U.S.A.* 82, 3197-3201.
- 30 Feyzi, E., Trybala, E., Bergstrom, T., Lindahl, U., and Spillmann, D. (1997). Structural requirement of heparan sulfate for interaction with herpes simplex virus type 1 virions and isolated glycoprotein C. *J.Biol.Chem.* 272, 24850-24857.
- Geraghty, R. J., Krummenacher, C., Cohen, G. H., Eisenberg, R. J., and Spear, P. G. (1998). Entry of alpha herpesviruses mediated by poliovirus receptor-related protein 1 and poliovirus receptor. *Science* 280, 1618-1620.
- 35 Herold, B. C., Gerber, S. I., Belval, B. J., Siston, A. M., and Shulman, N. (1996). Differences in the susceptibility of herpes simplex virus types 1 and 2 to modified heparin compounds suggest serotype differences in viral entry. *J.Virol.* 70, 3461-3469.
- 40 Herold, B. C., Visalli, R. J., Susmarski, N., Brandt, C. R., and Spear, P. G. (1994). Glycoprotein C-independent binding of herpes simplex virus to cells requires of cell surface heparan sulphate and glycoprotein B. *J.Gen.Virol.* 75, 1211-1222.

- Herold, B. C., WuDunn, D., Soltys, N., and Spear, P. G. (1991). Glycoprotein C of herpes simplex virus type 1 plays a principal role in the adsorption of virus to cells and in infectivity. *J.Virol.* 65, 1090-1098.
- Isola, V. J., Eisenberg, R. J., Siebert, G. R., Heilman, C. J., Wilcox, W. C., and Cohen, G. H. (1989). Fine mapping of antigenic site II of herpes simplex virus glycoprotein D. *J.Virol.* 63, 2325-2334.
- Jackson, T., Ellard, F. M., Ghazaleh, R. A., Brookes, S. M., Blakemore, W. E., Corteyn, A. H., Stuart, D. I., Newman, J. W., and King, A. M. (1996). Efficient infection of cells in culture by type O foot-and-mouth disease virus requires binding to cell surface heparan sulfate. *J.Virol.* 70, 5282-5287.
- Kakuta, Y., Sueyoshi, T., Negishi, M., and Pedersen, L. C. (1999). Crystal structure of the sulfotransferase domain of human heparan sulfate N-deacetylase/N-sulfotransferase 1. *J.Biol.Chem.* 274, 10673-10676.
- Krummenacher, C., Nicola, A. V., Whitbeck, J. C., Lou, H., Hou, W., Lambris, J. D., Geraghty, R. J., Spear, P. G., Cohen, G. H., and Eisenberg, R. J. (1998). Herpes simplex virus glycoprotein D can bind to poliovirus receptor-related protein 1 or herpesvirus entry mediator, two structurally unrelated mediators of virus entry. *J.Virol.* 72, 7064-7074.
- Krummenacher, C., Rux, A. H., Whitbeck, J. C., Ponce de Leon, M., Lou, H., Baribaud, I., Hou, W., Zou, C., Geraghty, R. J., Spear, P. G., Eisenberg, R. J., and Cohen, G. H. (1999). The first Ig-like domain of HveC is sufficient to bind HSV gD with full affinity while the third domain is involved in oligomerization of HveC. *J.Virol.* in press.
- Lafferty, W. E., Coombs, R. W., Benedetti, J., Critchlow, C., and Corey, L. (1987). Recurrences after oral and genital herpes simplex virus infection. Influence of site of infection and viral type. *N Engl J Med* 316, 1444-1449.
- Lee, M. K., and Lander, A. D. (1991). Analysis of affinity and structural selectivity in the binding of proteins to glycosaminoglycans: Development of a sensitive electrophoretic approach. *Proc.Natl.Acad.Sci.U.S.A.* 88, 2768-2772.
- Lidholt, K., Weinke, J. L., Kiser, C. S., Lugenwa, F. N., Bame, K. J., Cheifetz, S., Massagué, J., Lindahl, U., and Esko, J. D. (1992). A single mutation affects both N-acetylglucosaminyl-transferase and glucuronosyltransferase activities in a Chinese hamster ovary cell mutant defective in heparan sulfate biosynthesis. *Proc.Natl.Acad.Sci.U.S.A.* 89, 2267-2271.
- Lindahl, U., Kusche-Gullberg, M., and Kjellén, L. (1998). Regulated diversity of heparan sulfate. *J.Biol.Chem.* 273, 24979-24982.
- Liu, J., Shworak, N. W., Fritze, L. M. S., Edelberg, J. M., and Rosenberg, R. D. (1996). Purification of heparan sulfate D-glucosaminyl 3-O-sulfotransferase. *J.Biol.Chem.* 271, 27072-27082.
- Liu, J., Shworak, N. W., Sinay, P., Schwartz, J. J., Zhang, L., Fritze, L. M. S., and Rosenberg, R. (1999a). Expression of heparan sulfate D-glucosaminyl 3-O-sulfotransferase isoforms reveals novel substrate specificities. *J.Biol.Chem.* 274, 5185-5192.
- Lui, J., Shriver, Z., Blaiklock, P., Yoshida, K., Sasisekharan, R., and Rosenberg, R.D., (1999b) Heparan Sulfate D-glucoaminyl 3-O-sulfotransferase-3A sulfates N-unsubstituted glucosamine residues. *J.Biol.Chem.* 274, 38155-38162.

- Lopez, M., Eberlé, F., Mattei, M. G., Gabert, J., Birg, F., Bardin, F., Maroc, C., and Dubreuil, P. (1995). Complementary DNA characterization and chromosomal localization of a human gene related to the poliovirus receptor-encoding gene. *Gene* 155, 261-265.
- 5 Marsolais, F., and Varin, L. (1995). Identification of amino acid residues critical for catalysis and cosubstrate binding in the flavonol 3-sulfotransferase. *J.Biol.Chem.* 270, 30458-30463.
- Mauri, D. N., Ebner, R., Montgomery, R. I., Kochel, K. D., Cheung, T. C., Yu, G.-L., Ruben, S., Murphy, M., Eisenberg, R. J., Cohen, G. H., Spear, P. G., and Ware, C. F. (1998). LIGHT, a new member of the TNF superfamily, and lymphotoxin α are ligands for herpesvirus entry mediator. *Immunity* 8, 21-30.
- 10 Mendelsohn, C. L., Wimmer, E., and Racaniello, V. R. (1989). Cellular receptor for poliovirus: molecular cloning, nucleotide sequence, and expression of a new member of the immunoglobulin superfamily. *Cell* 56, 855-865.
- Montgomery, R. I., Warner, M. S., Lum, B. J., and Spear, P. G. (1996). Herpes simplex virus-1 entry into cells mediated by a novel member of the TNF/NGF receptor family. *Cell* 87, 427-436.
- 15 Nicola, A. V., Willis, S. H., Naidoo, N. N., Eisenberg, R. J., and Cohen, G. H. (1996). Structure-function analysis of soluble forms of herpes simplex virus glycoprotein D. *J.Virol.* 70, 3815-3822.
- Rosenberg, R. D., Shworak, N. W., Liu, J., Schwartz, J. J., and Zhang, L. (1997). Heparan sulfate proteoglycans of the cardiovascular system. *J.Clin.Invest.* 100, S67-S75.
- 20 Schmidt, J., Klupp, B. G., Karger, A., and Mettenleiter, T. C. (1997). Adaptability in herpesviruses: glycoprotein D-independent infectivity of pseudorabies virus. *J Virol* 71, 17-24.
- Schroder, C., Linde, G., Fehler, F., and Keil, G. M. (1997). From essential to beneficial: glycoprotein D loses importance for replication of bovine herpesvirus 1 in cell culture. *Journal of Virology* 71, 25-33.
- 25 Shieh, M.-T., WuDunn, D., Montgomery, R. I., Esko, J. D., and Spear, P. G. (1992). Cell surface receptors for herpes simplex virus are heparan sulfate proteoglycans. *J.Cell Biol.* 116, 1273-1281.
- Shworak, N. W., Liu, J., Fritze, L. M. S., Schwartz, J. J., Zhang, L., Logeart, D., and Rosenberg, R. D. (1997). Molecular cloning and expression of mouse and human cDNAs encoding heparan sulfate D-glucosaminyl 3-O-sulfotransferase. *J.Biol.Chem.* 272, 28008-28019.
- 30 Shworak, N. W., Liu, J., Petros, L. M., Zhang, L., Kobayashi, M., Copeland, N. G., Jenkins, N. A., and Rosenberg, R. D. (1999). Multiple isoforms of heparan sulfate D-glucosaminyl 3-O-sulfotransferase. *J.Biol.Chem.* 274, 5170-5184.
- Spear, P. G. (1993). Entry of alpha herpesviruses into cells. *Sem.Virol.* 4, 167-180.
- 35 Sueyoshi, T., Kakuta, Y., Pedersen, L. C., Wall, F. E., Pedersen, L. G., and Negishi, M. (1998). A role of Lys614 in the sulfotransferase activity of human heparan sulfate N-deacetylase/N-sulfotransferase. *FEBS Lett* 433, 211-214.
- Summerford, C., Bartlett, J. S., and Samulski, R. J. (1999). α Vb5 integrin: a co-receptor for adeno-associated virus type 2 infection. *Nature Med.* 5, 78-82.
- 40 Summerford, C., and Samulski, R. J. (1998). Membrane-associated heparan sulfate proteoglycan is a receptor for adeno-associated virus type 2 virions. *J.Virol.* 72, 1438-1445.

Takahashi, K., Nakanishi, H., Miyahara, M., Mandai, K., Satoh, K., Satoh, A., Nishioka, H., Aoki, J., Nomoto, A., Mizoguchi, A., and Takai, Y. (1999). Nectin/PRR: an immunoglobulin-like cell adhesion molecule recruited to cadherin-based adherens junctions through interaction with afadin, a PDZ domain-containing protein. *J. Cell Biol.* 145, 539-549.

- 5 Tal-Singer R, Peng C, Ponce De Leon M, Abrams WR, Banfield BW, Tufaro F, Cohen GH, Eisenberg RJ (1995) Interaction of herpes simplex virus glycoprotein gC with mammalian cell surface molecules. *J. Virol.* 69(7):4471-83.

- Warner, M. S., Geraghty, R. J., Martinez, W. M., Montgomery, R. I., Whitbeck, J. C., Xu, R., Eisenberg, R. J., Cohen, G. H., and Spear, P. G. (1998). A cell surface protein with herpesvirus entry activity (HveB) confers susceptibility to infection by mutants of herpes simplex virus type 1, herpes simplex virus type 2 and pseudorabies virus. *Virology* 246, 179-189.

- 10 Whitbeck, J. C., Peng, C., Lou, H., Xu, R., Willis, S. H., Ponce de Leon, M., Peng, T., Nicola, A. V., Montgomery, R. I., Warner, M. S., Soulika, A. M., Spruce, L. A., Moore, W. T., Lambris, J. D., Spear, P. G., Cohen, G. H., and Eisenberg, R. J. (1997). Glycoprotein D of herpes simplex virus (HSV) binds directly to HVEM, a member of the tumor necrosis factor receptor superfamily and a mediator of HSV entry. *J. Virol.* 71, 6083-6093.

- 15 Willis, S. H., Rux, A. J., Peng, C., Whitbeck, J. C., Nicola, A. V., Lou, H., Hou, W., Salvador, L., Eisenberg, R. J., and Cohen, G. H. (1998). Binding kinetics of herpes simplex virus (HSV) glycoprotein D to the herpesvirus entry mediator (HVEM/HveA) using surface plasmon resonance. *J. Virol.* 72, 5937-5947.
- 20

CLAIMS

What is claimed is:

- 1 1. A pharmaceutical preparation for inhibiting herpes simplex virus type-1 (HSV-1)
2 infection in a mammal comprising a substantially pure polysaccharide preparation
3 enriched for 3-O-sulfated glucosamine residues.
- 1 2. The pharmaceutical preparation of claim 1, wherein said polysaccharide preparation is
2 enriched for GlcN3S6S residues.
- 1 3. The pharmaceutical preparation of claims 1 or 2, comprising a polysaccharide preparation
2 enriched for polysaccharide structures capable of specific binding with HSV-1 gD viral
3 glycoprotein.
- 1 4. The pharmaceutical preparation of claim 3, wherein said polysaccharide preparation
2 enriched for polysaccharide structures capable of specific binding with HSV-1 gD viral
3 glycoprotein has a binding affinity of less than 30 μM for gD.
- 1 5. The pharmaceutical preparation of claim 3, wherein said polysaccharide preparation
2 enriched for polysaccharide structures capable of specific binding with HSV-1 gD viral
3 glycoprotein has a binding affinity of less than 20 μM for gD.
- 1 6. The pharmaceutical preparation of claim 3, wherein said polysaccharide preparation
2 enriched for polysaccharide structures capable of specific binding with HSV-1 gD viral
3 glycoprotein has a binding affinity of less than 10 μM for gD.
- 1 7. The pharmaceutical preparation of claim 3, wherein said polysaccharide preparation
2 enriched for polysaccharide structures capable of specific binding with HSV-1 gD viral
3 glycoprotein has a binding affinity of 2 μM for gD.
- 1 8. The pharmaceutical preparation of claim 3, wherein said polysaccharide preparation
2 enriched for polysaccharide structures capable of specific binding with HSV-1 gD viral
3 glycoprotein has a binding affinity of about 2 μM to about 10 μM for gD.
- 1 9. A pharmaceutical preparation for inhibiting herpes simplex virus type-1 (HSV-1)
2 infection in a mammal, comprising heparan sulfate that has been enriched for
3 polysaccharide structures modified by a 3-OST-3 enzyme.

- 1 10. A pharmaceutical preparation as in claim 9, wherein said pharmaceutical preparation has
2 been enriched by contacting said polysaccharide with 3-OST-3 enzyme in the presence of
3 a sulfate donor, under conditions suitable for 3-O sulfation of said polysaccharide by 3-
4 OST-3.
- 1 11. A pharmaceutical preparation as in claim 10 wherein the 3-OST-3 enzyme is selected
2 from the group consisting of 3-OST-3A and 3-OST-3B.
- 1 12. The pharmaceutical preparation of claims 1-11, wherein the preparation comprises the
2 disaccharide sequence -IdoA2S-GlcN3S6S.
- 1 13. The pharmaceutical preparation of claims 1-11 wherein the preparation comprises the
2 trisaccharide sequence GlcNS-IdoA2S-GlcNH₂3S6S.
- 1 14. The pharmaceutical preparation of claims 1-11 wherein the preparation comprises the
2 tetrasaccharide sequence UA2S-GlcNS-IdoA2S-GlcNH₂3S6S.
- 1 15. The pharmaceutical preparation of claims 1-11 wherein the preparation comprises the
2 pentasaccharide sequence GlcNS6S-UA2S-GlcNS-IdoA2S-GlcNH₂3S6S.
- 1 16. The pharmaceutical preparation of claims 1-11 wherein the preparation comprises the
2 hexasaccharide sequence UA-GlcNS6S-UA2S-GlcNS-IdoA2S-GlcNH₂3S6S.
- 1 17. The pharmaceutical preparation of claims 1-11 wherein the preparation comprises the
2 hexasaccharide sequence UA-GlcNS6S-UA2S-GlcNS-IdoA2S-GlcNH₂3S6S.
- 1 18. The pharmaceutical preparation of claims 1-11 wherein the preparation comprises the
2 heptasaccharide sequence GlcNAc-UA-GlcNS6S-UA2S-GlcNS-IdoA2S-GlcNH₂3S6S.
- 1 19. The pharmaceutical preparation of claims 1-11 wherein the preparation comprises the
2 octasaccharide sequence UA-GlcNAc-UA-GlcNS6S-UA2S-GlcNS-IdoA2S-
3 GlcNH₂3S6S.
- 1 20. The pharmaceutical preparation of claims 1-19, wherein said pharmaceutical preparation
2 comprises pharmaceutically acceptable carrier selected from the group consisting of
3 lotions, creams, jellies, liniments, ointments, salves, oils, foams, gels, washes,
4 suppositories, slow-releasing polymers, and coatings.

- 1 21. The pharmaceutical preparation of claims 1-20, wherein said pharmaceutical preparation
2 further comprises at least one skin penetrating enhancer.
- 1 22. The pharmaceutical preparation of claim 1-21, wherein said skin penetrating enhancer is
2 selected from the group consisting of dimethylsulfoxide (DMSO), propylene glycol,
3 isopropanol, ethanol, oleic acid, and N-methylpyrrolidone.
- 1 23. A method of inhibiting herpes simplex virus type-1 (HSV-1) viral infection in mammal
2 comprising administering a therapeutically effective amount of any one of the
3 pharmaceutical preparations of claims 1-22 to a mammal at risk of HSV-1 infection.
- 1 24. A method of inhibiting herpes simplex virus type-1 (HSV-1) viral infection in mammal
2 comprising administering a therapeutically effective amount of any one of the
3 pharmaceutical preparations of claims 1-22 to a mammal diagnosed with HSV-1
4 infection.

(12) INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(19) World Intellectual Property Organization
International Bureau



(43) International Publication Date
1 March 2001 (01.03.2001)

PCT

(10) International Publication Number
WO 01/13910 A2

- (51) International Patent Classification⁷: **A61K 31/00**
- (21) International Application Number: **PCT/US00/23288**
- (22) International Filing Date: **25 August 2000 (25.08.2000)**
- (25) Filing Language: **English**
- (26) Publication Language: **English**
- (30) Priority Data:
60/150,743 25 August 1999 (25.08.1999) US
60/175,347 10 January 2000 (10.01.2000) US
- (71) Applicants (for all designated States except US):
MASSACHUSETTS INSTITUTE OF TECHNOLOGY [US/US]; 77 Massachusetts Avenue, Cambridge, MA 02138 (US). NORTHWESTERN UNIVERSITY [US/US]; 633 Clark Street, Evanston, IL 60208 (US).
- (72) Inventors; and
- (75) Inventors/Applicants (for US only): **SHUKLA, Deepak [IN/US]; 8339 Kimball Avenue, Skokie, IL 60076 (US). LIU, Jian [CN/US]; 106 Ivy Brook Lane, Chapel Hill, NC 27516 (US). ROSENBERG, Robert, D. [US/US]; Apartment 3, 28 Commonwealth Avenue, Boston, MA 02116 (US). SPEAR, Patricia, G. [US/US]; 5719 S. Kenwood, Chicago, IL 60637 (US).**
- (74) Agent: **BRESNAHAN, Maureen, A.; Testa, Hurwitz & Thibault, LLP, High Street Tower, 125 High Street, Boston, MA 02110 (US).**
- (81) Designated States (national): **AE, AG, AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, BZ, CA, CH, CN, CR, CU, CZ, DE, DK, DM, DZ, EE, ES, FI, GB, GD, GE, GH, GM, HR, HU, ID, IL, IN, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MA, MD, MG, MK, MN, MW, MX, MZ, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, SL, TJ, TM, TR, TT, TZ, UA, UG, US, UZ, VN, YU, ZA, ZW.**
- (84) Designated States (regional): **ARIPO patent (GH, GM, KE, LS, MW, MZ, SD, SL, SZ, TZ, UG, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, GW, ML, MR, NE, SN, TD, TG).**
- Published:**
— Without international search report and to be republished upon receipt of that report.
- For two-letter codes and other abbreviations, refer to the "Guidance Notes on Codes and Abbreviations" appearing at the beginning of each regular issue of the PCT Gazette.*

(54) Title: **PHARMACEUTICAL PREPARATIONS FOR THE INHIBITION OF HERPES SIMPLEX VIRUS 1 ENTRY**

(57) Abstract: Disclosed herein are polysaccharide preparations enriched in 3-OST-3 modified heparan sulfate. Also disclosed are methods of treating herpes simplex viral type-1 infection using the pharmaceutical preparations of the invention.

WO 01/13910 A2

1/2

m3-OST-3B (1) MGQRLSGGRSCLDVPGRFLPQPPPPPPVRRKLALLFAMLCIWL^VYMFLYSCAGSCTAAPG
 h3-OST-3B (1)L.....V.....A....

 m3-OST-3B (61) LLLLGSGSRATHAQPTLV^VTAPNETSPKMPFRAPPANSLAAGKDKTVGAGSQEEQSPEAPD
 h3-OST-3B (61)A.DP.A.A...DG.P.RL.....TP..S..EMAE..A.P.....V..

 m3-OST-3B (121) SPSPISFFSGAGSKQLPQAIIGVKKGGTRALLEFLRVHPDVRAVGAEPHFFDRSYHKG
 h3-OST-3B (121)S.....K.....D..
 h3-OST-3A (148)D.....D..

 m3-OST-3B (181) LAWYRDLMRPTLKGQITMEKTPSYFVTREAPARISAMSKDTKLI^VVVRDPVTRAISDYTQ
 h3-OST-3B (181)D.....
 h3-OST-3A (196)D.....

 m3-OST-3B (241) TLSKRPDIPSFESLTFNRNSAGLIDTSWSAIIQIGLYAKHLEPWL^RHFPLGQMLFVSGERL
 h3-OST-3B (241)T....K..T.....I.....H.....IR.....
 h3-OST-3A (256)T....K..T.....I.....H.....IR.....

 m3-OST-3B (301) VSDPAGELRRVQDFLGLKRIITDKHFFYENOTKGFPC^LLKKAEGSGKPHCLGKTKGRAHPTI
 h3-OST-3B (301) I.....G.....K.....SR.....T..E.
 h3-OST-3A (316) I.....G.....K.....SR.....T..E.

 m3-OST-3B (361) AREVLRQLRDFYRPFNRK^FYQMTGRDFGWD-
 h3-OST-3B (361) D...V.R..E.....L.....H.....
 h3-OST-3A (376) D...V.R..E.....L.....H.....G

FIG. 1

2/2

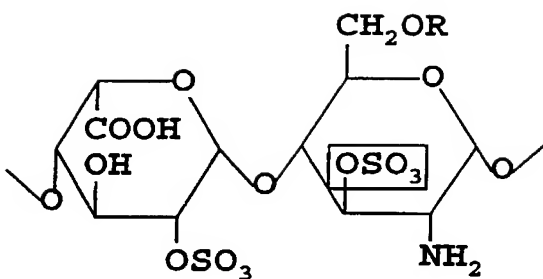


FIG. 2

DECLARATION AND POWER OF ATTORNEY FOR UTILITY OR DESIGN PATENT APPLICATION	Attorney Docket No.	MIT-108
	First Named Inventor	Shukla
	COMPLETE IF KNOWN	
	Application Serial Number	10/069,280
	Filing Date	February 25, 2002
	Group Art Unit	Not yet assigned
<input type="checkbox"/> Declaration <input checked="" type="checkbox"/> Declaration Submitted with Submitted after Initial Initial Filing Filing (surcharge 37 CFR 1.16(e) required)	Examiner Name	Not yet assigned

As a below named inventor, I hereby declare that:

My residence, mailing address, and citizenship are as stated below next to my name.

I believe I am the original, first and sole inventor (if only one name is listed below) or an original, first and joint inventor (if plural names are listed below) of the subject matter which is claimed and for which a patent is sought on the invention entitled:

PHARMACEUTICAL PREPARATIONS FOR THE INHIBITION OF HERPES SIMPLEX VIRUS 1 ENTRY

(Title of the Invention)

the specification of which

☐ is attached hereto
OR

☒ was filed on February 25, 2002 as United States Application Serial Number or PCT International (MM/DD/YYYY)

Application Number 10/069,280 and was amended on (MM/DD/YYYY) (if applicable)

I hereby state that I have reviewed and understand the contents of the above-identified application, including the claims, as amended by any amendment specifically referred to above.

I acknowledge the duty to disclose to the Patent Office all information known by me to be material to patentability as defined in 37 CFR 1.56, including for continuation-in-part applications, material information known by me which became available between the filing date of the prior application and the national or PCT international filing date of the continuation-in-part application.

I hereby claim foreign priority benefits under 35 U.S.C. 119(a)-(d) or (f), or 365(b) of any foreign application(s) for patent, or inventor's certificate(s), or 365(a) of any PCT international application which designated at least one country other than the United States of America, listed below and have also identified below, by checking the box, any foreign application for patent, or inventor's certificate(s), or of any PCT international application having a filing date before that of the application on which priority is claimed.

Prior Foreign Application Number(s)	Country	Foreign Filing Date (MM/DD/YYYY)	Priority Not Claimed	Certified Copy Attached?	
				YES	NO
PCT/US00/23288	WO	08/25/2000	<input type="checkbox"/> <input type="checkbox"/> <input type="checkbox"/> <input type="checkbox"/>	<input type="checkbox"/> <input type="checkbox"/> <input type="checkbox"/> <input type="checkbox"/>	<input type="checkbox"/> <input type="checkbox"/> <input type="checkbox"/> <input type="checkbox"/>

☐ Additional foreign application numbers are listed on a supplemental priority data sheet attached hereto.

I hereby claim the benefit under 35 U.S.C. 119(e) of any United States provisional application(s) listed below.

Application Serial Number(s)	Filing Date (MM/DD/YYYY)	<input type="checkbox"/> Additional provisional application serial numbers are listed on a supplemental priority data sheet attached hereto.
60/150,753	08/25/1999	
60/175,347	01/10/2000	

DECLARATION – Utility or Design Patent Application

I hereby claim the benefit under 35 U.S.C. 120 of any United States application(s), or 365(c), of any PCT international application designating the United States of America, listed below and, insofar as the subject matter of each of the claims of this application is not disclosed in the prior United States or PCT International application in the manner provided by the first paragraph of 35 U.S.C. 112, I acknowledge the duty to disclose information which is material to patentability as defined in 37 CFR 1.56 which became available between the filing date of the prior application and the national or PCT international filing date of this application

U.S. Parent Application or PCT Parent Serial Number	Parent Filing Date (MM/DD/YYYY)	Parent Patent Number (if applicable)

☐ Additional U.S. or PCT international application numbers are listed on a supplemental priority data sheet attached hereto.

As a named inventor, I hereby appoint the following registered practitioners to prosecute this application and to transact all business in the Patent and Trademark Office connected therewith: ☐ Customer Number

OR

☒ Registered practitioner(s) name/registration number listed below

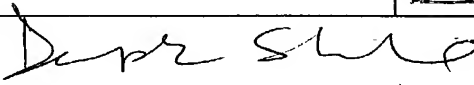
Place Customer
Number Bar Code
Label Here

Name	Registration Number	Name	Registration Number
Michael J. Bastian	47,411	John D. Lanza	40,060
Steven M. Bauer	31,481	Kurt W. Lockwood	40,704
Elias C. Behrakis	47,416	Leigh J. Martinson	50,749
Mark L. Beloborodov	50,773	Thomas C. Meyers	36,989
John V. Bianco	36,748	Joseph B. Milstein	42,897
Robert S. Blasi	50,389	David G. Miranda	42,898
Maureen A. Bresnahan	44,559	Ronda P. Moore	44,244
Michael H. Brodowski	41,640	Jeremy Oczek	50,794
Jennifer A. Camacho	43,526	Edmund R. Pitcher	27,829
Joseph A. Capraro, Jr.	36,471	Jamie H. Rose	45,054
Christopher H. Chung	50,351	David L. Schuler	51,190
John J. Cotter	38,116	Christopher W. Stamos	35,370
Robert V. Donahoe	46,667	Diana M. Steel	43,153
Brian A. Fairchild	48,645	Joseph P. Sullivan	45,349
John V. Forcier	42,545	Robert J. Tosti	35,393
Steven J. Frank	33,497	Thomas A. Turano	35,722
Kia L. Freeman	47,577	Christine C. Vito	39,061
Brian M. Gaff	44,691	Patrick R.H. Waller	41,418
Duncan A. Greenhalgh	38,678	Daniel A. Wilson	45,508
Ira V. Heffan	41,059	Gerald E. Worth	45,238
Danielle L. Herriott	43,670	Yin P. Zhang	44,372
Douglas J. Kline	35,574		
<input type="checkbox"/> Additional registered practitioners named on supplemental Registered Practitioner Information sheet attached hereto.			

Direct all correspondence to:

Patent Administrator
 Testa, Hurwitz & Thibault, LLP
 High Street Tower
 125 High Street
 Boston, MA 02110
 Tel No.: (617) 248-7000
 Fax No.: (617) 248-7100

I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under 18 U.S.C. 1001 and that such willful false statements may jeopardize the validity of the application or any patent issued thereon.

Name of Sole or First Inventor:		<input type="checkbox"/> A petition has been filed for this unsigned inventor							
Given Name (first and middle [if any])						Family Name or Surname			
Deepak						Shukla			
Inventor's Signature						Date		04/30/2002	
Residence	City	Skokie	State	IL	Country	US	Citizenship	China INDIA	
Mailing Address		8339 Kimball Avenue							
Mailing Address (In. 2)	City	Skokie	State	IL	ZIP	60076	Country	US	
<input type="checkbox"/> Additional inventors are being named on the supplemental Additional Inventor(s) sheet(s) attached hereto.									
Name of Additional Joint Inventor, if any:		<input type="checkbox"/> A petition has been filed for this unsigned inventor							
Given Name (first and middle [if any])						Family Name or Surname			
Robert D.						Rosenberg			
Inventor's Signature						Date			
Residence	City	Boston	State	MA	Country	US	Citizenship	US	
Mailing Address		28 Commonwealth Avenue							
Mailing Address (In. 2)	City	Boston	State	MA	ZIP	02116	Country	US	
Name of Additional Joint Inventor, if any:		<input type="checkbox"/> A petition has been filed for this unsigned inventor							
Given Name (first and middle [if any])						Family Name or Surname			
Patricia G.						Spear			
Inventor's Signature						Date			
Residence	City	Chicago	State	IL	Country	US	Citizenship	US	
Mailing Address		5719 S. Kenwood							
Mailing Address (In. 2)	City	Chicago	State	IL	ZIP	60637	Country	US	

DECLARATION AND POWER OF ATTORNEY FOR UTILITY OR DESIGN PATENT APPLICATION <input type="checkbox"/> Declaration <input checked="" type="checkbox"/> Declaration Submitted with Submitted after Initial Initial Filing Filing (surcharge 37 CFR 1.16(c) required)	Attorney Docket No.	MIT-108
	First Named Inventor	Shukla
	COMPLETE IF KNOWN	
	Application Serial Number	10/069,280
	Filing Date	February 25, 2002
	Group Art Unit	Not yet assigned
	Examiner Name	Not yet assigned

As a below named inventor, I hereby declare that:

My residence, mailing address, and citizenship are as stated below next to my name.

I believe I am the original, first and sole inventor (if only one name is listed below) or an original, first and joint inventor (if plural names are listed below) of the subject matter which is claimed and for which a patent is sought on the invention entitled:

PHARMACEUTICAL PREPARATIONS FOR THE INHIBITION OF HERPES SIMPLEX VIRUS 1 ENTRY

(Title of the Invention)

the specification of which

☐ is attached hereto

OR

☒ was filed on

February 25, 2002

as United States Application Serial Number or PCT International

Application Number

10/069,280

and was amended on (MM/DD/YYYY) (if applicable)

I hereby state that I have reviewed and understand the contents of the above-identified application, including the claims, as amended by any amendment specifically referred to above.

I acknowledge the duty to disclose to the Patent Office all information known by me to be material to patentability as defined in 37 CFR 1.56, including for continuation-in-part applications, material information known by me which became available between the filing date of the prior application and the national or PCT international filing date of the continuation-in-part application.

I hereby claim foreign priority benefits under 35 U.S.C. 119(a)-(d) or (f), or 365(b) of any foreign application(s) for patent, or inventor's certificate(s), or 365(a) of any PCT international application which designated at least one country other than the United States of America, listed below and have also identified below, by checking the box, any foreign application for patent, or inventor's certificate(s), or of any PCT international application having a filing date before that of the application on which priority is claimed.

Prior Foreign Application Number(s)	Country	Foreign Filing Date (MM/DD/YYYY)	Priority Not Claimed	Certified Copy Attached?	
PCT/US00/23288	WO	08/25/2000	<input type="checkbox"/>	YES <input type="checkbox"/>	NO <input type="checkbox"/>
			<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>
			<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>
			<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>

☐ Additional foreign application numbers are listed on a supplemental priority data sheet attached hereto.

I hereby claim the benefit under 35 U.S.C. 119(e) of any United States provisional application(s) listed below.

Application Serial Number(s)	Filing Date (MM/DD/YYYY)	<input type="checkbox"/> Additional provisional application serial numbers are listed on a supplemental priority data sheet attached hereto
60/150,753	08/25/1999	
60/175,347	01/10/2000	

Declaration and Power of Attorney for Utility or Design Patent Application

Serial No. 10/069,280

Atty. Docket No. MIT-108

Page 2 of 3

DECLARATION – Utility or Design Patent Application

I hereby claim the benefit under 35 U.S.C. 120 of any United States application(s), or 365(c), of any PCT international application designating the United States of America, listed below and, insofar as the subject matter of each of the claims of this application is not disclosed in the prior United States or PCT International application in the manner provided by the first paragraph of 35 U.S.C. 112, I acknowledge the duty to disclose information which is material to patentability as defined in 37 CFR 1.56 which became available between the filing date of the prior application and the national or PCT international filing date of this application.

U.S. Parent Application or PCT Parent Serial Number	Parent Filing Date (MM/DD/YYYY)	Parent Patent Number (if applicable)

☐ Additional U.S. or PCT international application numbers are listed on a supplemental priority data sheet attached hereto.

As a named inventor, I hereby appoint the following registered practitioners to prosecute this application and to transact all business in the Patent and Trademark Office connected therewith. ☐ Customer Number

OR

☒ Registered practitioner(s) name/registration number listed below

Place Customer
Number Bar Code
Label Here

Name	Registration Number	Name	Registration Number
Michael J. Bastian	47,411	John D. Lanza	40,060
Steven M. Bauer	31,481	Kurt W. Lockwood	40,704
Elias C. Behrakis	47,416	Leigh J. Martinson	50,749
Mark L. Beloborodov	50,773	Thomas C. Meyers	36,989
John V. Bianco	36,748	Joseph B. Milstein	42,897
Robert S. Blasi	50,389	David G. Miranda	42,898
Maureen A. Bresnahan	44,559	Ronda P. Moore	44,244
Michael H. Brodowski	41,640	Jeremy Occek	50,794
Jennifer A. Camacho	43,526	Edmund R. Pitcher	27,829
Joseph A. Capraro, Jr.	36,471	Jamie H. Rose	45,054
Christopher H. Chung	50,351	David L. Schuler	51,190
John J. Cotter	38,116	Christopher W. Stamos	35,370
Robert V. Donahoe	46,667	Diana M. Steel	43,153
Brian A. Fairchild	48,645	Joseph P. Sullivan	45,349
John V. Forcier	42,545	Robert J. Tosti	35,393
Steven J. Frank	33,497	Thomas A. Turano	35,722
Kia L. Freeman	47,577	Christine C. Vito	39,061
Brian M. Gaff	44,691	Patrick R.H. Waller	41,418
Duncan A. Greenhalgh	38,678	Daniel A. Wilson	45,508
Ira V. Heffan	41,059	Gerald E. Worth	45,238
Danielle L. Herritt	43,670	Yin P. Zhang	44,372
Douglas J. Kline	35,574		
<input type="checkbox"/> Additional registered practitioners named on supplemental Registered Practitioner Information sheet attached hereto.			

Direct all correspondence to:

Patent Administrator
Testa, Hurwitz & Thibault, LLP
High Street Tower
125 High Street
Boston, MA 02110
Tel. No.: (617) 248-7000
Fax No.: (617) 248-7100

Declaration and Power of Attorney for Utility or Design Patent Application
 Serial No. 10/069,280
 Atty. Docket No. MIT-108
 Page 3 of 3

I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true, and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under 18 U.S.C. 1001 and that such willful false statements may jeopardize the validity of the application or any patent issued thereon.

Name of Sole or First Inventor:		<input type="checkbox"/> A petition has been filed for this unsigned inventor					
Given Name (first and middle [if any])				Family Name or Surname			
Deepak				Shukla			
Inventor's Signature						Date	
Residence	City	Skokie	State	IL	Country	US	Citizenship
Mailing Address		8339 Kimball Avenue					
Mailing Address (ln. 2)	City	Skokie	State	IL	ZIP	60076	Country
						US	
<input type="checkbox"/> Additional inventors are being named on the supplemental Additional Inventor(s) sheet(s) attached hereto.							
Name of Additional Joint Inventor, if any:		<input type="checkbox"/> A petition has been filed for this unsigned inventor					
Given Name (first and middle [if any])				Family Name or Surname			
Robert D.				Rosenberg			
Inventor's Signature		<i>Robert D. Rosenberg</i>				Date	
						7/22/02	
Residence	City	Boston	State	MA	Country	US	Citizenship
Mailing Address		28 Commonwealth Avenue					
Mailing Address (ln. 2)	City	Boston	State	MA	ZIP	02116	Country
						US	
Name of Additional Joint Inventor, if any:		<input type="checkbox"/> A petition has been filed for this unsigned inventor					
Given Name (first and middle [if any])				Family Name or Surname			
Patricia G.				Spear			
Inventor's Signature						Date	
Residence	City	Chicago	State	IL	Country	US	Citizenship
Mailing Address		5719 S. Kenwood					
Mailing Address (ln. 2)	City	Chicago	State	IL	ZIP	60637	Country
						US	

DECLARATION AND POWER OF ATTORNEY FOR UTILITY OR DESIGN PATENT APPLICATION <input type="checkbox"/> Declaration <input checked="" type="checkbox"/> Declaration Submitted with Submitted after Initial Initial Filing Filing (surcharge 37 CFR 1.16(e) required)	Attorney Docket No.	MIT-108
	First Named Inventor	Shukla
	COMPLETE IF KNOWN	
	Application Serial Number	10/069,280
	Filing Date	February 25, 2002
	Group Art Unit	Not yet assigned
	Examiner Name	Not yet assigned

As a below named inventor, I hereby declare that:

My residence, mailing address, and citizenship are as stated below next to my name.

I believe I am the original, first and sole inventor (if only one name is listed below) or an original, first and joint inventor (if plural names are listed below) of the subject matter which is claimed and for which a patent is sought on the invention entitled:

PHARMACEUTICAL PREPARATIONS FOR THE INHIBITION OF HERPES SIMPLEX VIRUS 1 ENTRY

(Title of the Invention)

the specification of which

☐ is attached hereto
OR
☒ was filed on February 25, 2002 as United States Application Serial Number or PCT International (MM/DD/YYYY)

Application Number 10/069,280 and was amended on (MM/DD/YYYY) (if applicable).

I hereby state that I have reviewed and understand the contents of the above-identified application, including the claims, as amended by any amendment specifically referred to above.

I acknowledge the duty to disclose to the Patent Office all information known by me to be material to patentability as defined in 37 CFR 1.56, including for continuation-in-part applications, material information known by me which became available between the filing date of the prior application and the national or PCT international filing date of the continuation-in-part application.

I hereby claim foreign priority benefits under 35 U.S.C. 119(a)-(d) or (f), or 365(b) of any foreign application(s) for patent, or inventor's certificate(s), or 365(a) of any PCT international application which designated at least one country other than the United States of America, listed below and have also identified below, by checking the box, any foreign application for patent, or inventor's certificate(s), or of any PCT international application having a filing date before that of the application on which priority is claimed.

Prior Foreign Application Number(s)	Country	Foreign Filing Date (MM/DD/YYYY)	Priority Not Claimed	Certified Copy Attached?	
				YES	NO
PCT/US00/23288	WO	08/25/2000	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>
			<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>
			<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>
			<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>

☐ Additional foreign application numbers are listed on a supplemental priority data sheet attached hereto.

I hereby claim the benefit under 35 U.S.C. 119(e) of any United States provisional application(s) listed below.

Application Serial Number(s)	Filing Date (MM/DD/YYYY)	<input type="checkbox"/> Additional provisional application serial numbers are listed on a supplemental priority data sheet attached hereto.
60/150,753	08/25/1999	
60/175,347	01/10/2000	

DECLARATION – Utility or Design Patent Application

I hereby claim the benefit under 35 U.S.C. 120 of any United States application(s), or 365(c), of any PCT international application designating the United States of America, listed below and, insofar as the subject matter of each of the claims of this application is not disclosed in the prior United States or PCT International application in the manner provided by the first paragraph of 35 U.S.C. 112, I acknowledge the duty to disclose information which is material to patentability as defined in 37 CFR 1.56 which became available between the filing date of the prior application and the national or PCT international filing date of this application

U.S. Parent Application or PCT Parent Serial Number	Parent Filing Date (MM/DD/YYYY)	Parent Patent Number (if applicable)

☐ Additional U.S. or PCT international application numbers are listed on a supplemental priority data sheet attached hereto.

As a named inventor, I hereby appoint the following registered practitioners to prosecute this application and to transact all business in the Patent and Trademark Office connected therewith: ☐ Customer Number → Place Customer
Number Bar Code
Label Here

OR
☒ Registered practitioner(s) name/registration number listed below

Name	Registration Number	Name	Registration Number
Michael J. Bastian	47,411	John D. Lanza	40,060
Steven M. Bauer	31,481	Kurt W. Lockwood	40,704
Elias C. Behrakis	47,416	Leigh J. Martinson	50,749
Mark L. Beloborodov	50,773	Thomas C. Meyers	36,989
John V. Bianco	36,748	Joseph B. Milstein	42,897
Robert S. Blasi	50,389	David G. Miranda	42,898
Maureen A. Bresnahan	44,559	Ronda P. Moore	44,244
Michael H. Brodowski	41,640	Jeremy Oczek	50,794
Jennifer A. Camacho	43,526	Edmund R. Pitcher	27,829
Joseph A. Capraro, Jr.	36,471	Jamie H. Rose	45,054
Christopher H. Chung	50,351	David L. Schuler	51,190
John J. Cotter	38,116	Christopher W. Stamos	35,370
Robert V. Donahoe	46,667	Diana M. Steel	43,153
Brian A. Fairchild	48,645	Joseph P. Sullivan	45,349
John V. Forcier	42,545	Robert J. Tosti	35,393
Steven J. Frank	33,497	Thomas A. Turano	35,722
Kia L. Freeman	47,577	Christine C. Vito	39,061
Brian M. Gaff	44,691	Patrick R.H. Waller	41,418
Duncan A. Greenhalgh	38,678	Daniel A. Wilson	45,508
Ira V. Heffan	41,059	Gerald E. Worth	45,238
Danielle L. Herritt	43,670	Yin P. Zhang	44,372
Douglas J. Kline	35,574		
<input type="checkbox"/> Additional registered practitioners named on supplemental Registered Practitioner Information sheet attached hereto.			

Direct all correspondence to:

Patent Administrator
 Testa, Hurwitz & Thibault, LLP
 High Street Tower
 125 High Street
 Boston, MA 02110
 Tel. No.: (617) 248-7000
 Fax No.: (617) 248-7100

Declaration and Power of Attorney for Utility or Design Patent Application

Serial No. 10/069,280

Atty. Docket No. MIT-108

Page 3 of 3

I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under 18 U.S.C. 1001 and that such willful false statements may jeopardize the validity of the application or any patent issued thereon.

Name of Sole or First Inventor:		<input type="checkbox"/> A petition has been filed for this unsigned inventor								
Given Name (first and middle [if any])					Family Name or Surname					
Deepak					Shukla					
Inventor's Signature							Date			
Residence	City	Skokie	State	IL	Country	US	Citizenship	China		
Mailing Address		8339 Kimball Avenue								
Mailing Address (In. 2)	City	Skokie	State	IL	ZIP	60076	Country	US		
<input type="checkbox"/> Additional inventors are being named on the _____ supplemental Additional Inventor(s) sheet(s) attached hereto.										
Name of Additional Joint Inventor, if any:		<input type="checkbox"/> A petition has been filed for this unsigned inventor								
Given Name (first and middle [if any])					Family Name or Surname					
Robert D.					Rosenberg					
Inventor's Signature							Date			
Residence	City	Boston	State	MA	Country	US	Citizenship	US		
Mailing Address		28 Commonwealth Avenue								
Mailing Address (In. 2)	City	Boston	State	MA	ZIP	02116	Country	US		
Name of Additional Joint Inventor, if any:		<input type="checkbox"/> A petition has been filed for this unsigned inventor								
Given Name (first and middle [if any])					Family Name or Surname					
Patricia G.					Spear					
Inventor's Signature		<i>Patricia G. Spear</i>					Date		5/2/02	
Residence	City	Chicago	State	IL	Country	US	Citizenship	US		
Mailing Address		5719 S. Kenwood 505 N. Lake Shore Dr., # 3406 (Pgs)								
Mailing Address (In. 2)	City	Chicago	State	IL	ZIP	60637 60611	Country	US		

(Pgs)